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Studies of Translational Factors EF-G and EF-P

by

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in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biochemistry

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ABSTRACT

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ABSTRACT

The ribosome is the ribonucleoprotein machinery that orchestrates protein synthesis, and in turn, cell survival and adaptability. The ribosome functions synchronously with a plethora of protein factors. The focus of my thesis is on Elongation Factor G (EF-G), which catalyzes translocation during protein synthesis, and Elongation Factor P (EF-P), which promotes the formation of the first peptide bond.

Our EF-G work is focused on gaining insight into the role of the two “switch” regions, which derive energy from ribosome activated GTP hydrolysis to unlock the unidirectional progression of polypeptide chain elongation. Upon GTP hydrolysis, the switch I region of EF-G flips outside of the ribosome, and in doing so facilitates in nucleotide release and EF-G recycling. Conversely, switch II does not undergo extensive movements during elongation, but rather functions as a pivot at the interface between domains I and III, similar to a “ball and socket” type of mechanism stabilizes the partial rotation and extension of the EF-G molecule, driving the progression from GTP hydrolysis into translocation.

Fusidic acid is an antibiotic that blocks protein synthesis by trapping EF-G on the ribosome. The antibiotic uses the outward movement of the switch I region to bind onto EF-G at the interface between domains I and III, where it makes direct contact with switch II. This region is known to harbor spontaneous fusidic acid resistant mutations, with the residue situated at the tip of switch II hosting the strongest fusidic acid resistant mutant isolated to date.

EF-P was investigated to establish whether it acts as an initiation or as an elongation factor in translation. Here we show that EF-P does not actively function in either phase of translation, and that it does not act cooperatively with EF-G during translocation, as previously inferred. Rather, our results suggest that EF-P functions at an intermediate step between initiation and elongation, probably by stabilizing the initiator tRNA on the ribosome.

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LIST OF ABBREVIATIONS

aa - aminoacyl

A site – ribosomal aminoacyl (acceptor) site

a.u. – arbitrary units

CTD – C terminal domain

DMS – dimethylsulphate

E site – ribosomal exit site

EF – elongation factor

F site – ribosomal factor binding site

GAC – GTPase activating center

GAP(F) – GTPase activating protein (factor)

GDPNP – guanosine 5' (β,γ -imido)triphosphate

GEF - GTPase exchange factor

IF – initiation factor

IPTG – isopropyl β -D-1-thiogalactopyranoside

mant-GTP/GDP – 2'/3'-O-(N'-methylantranilonyl)-GTP/GDP

MTF – methionyl transformylase

Ni-NTA – nickel-nitrilo triacetic acid

NTD – N terminal domain

P site – ribosomal peptidyl site

PAGE – polyacrylamide gel electrophoresis

PDB – protein database

Pi – inorganic phosphate

PNPase – polynucleotide phosphorylase

PPi – inorganic pyrophosphate

p.s.i. – pounds per square inch

PTC – peptidyl transferase center

r-proteins – ribosomal proteins

RF – release factor

RRF – ribosome recycling factor

S – Svedberg units

SD sequence – Shine Dalgarno sequence

sm FRET – single molecule fluorescence resonance energy transfer

SRL – α -sarcin ricin loop

WT – wild type

**I. BACTERIAL TRANSLATION, ANTIBIOTICS AND THESIS
OVERVIEW**

I.1. INTRODUCTION

The process of gene expression converts genetically encoded information in the DNA into the corresponding functional gene product. Gene expression is universally used in eukaryotes, prokaryotes and viruses to generate protein and RNA, the essential components of all biological processes in the cell.

Protein gene expression starts with the transcription of the DNA sequences into RNA by the RNA polymerase (RNAP) machinery. In bacteria, concomitantly with transcription, the ribosomal machinery translates the messenger RNA (mRNA) and assembles the corresponding amino acids delivered by transfer RNAs (tRNAs) into polypeptide chains. Once synthesized, the primary sequence protein chain is released from the ribosome while being folded into its corresponding tertiary structure. Due to the higher complexity of the eukaryotic systems, and taking into account cell compartmentalization, in eukaryotic organisms, nuclear DNA transcription is followed by RNA processing, maturation and export into the cytoplasm, with subsequent more complex mechanisms of translation, protein folding and transport than in prokaryotes.

I.1.1 The bacterial ribosome

The research community started showing interest in examining the cellular content in the early 1800s, with the visualization of the nucleus and of its chromosomes by Robert Brown and Walther Flemming, respectively. In the next century, as the resolution of microscopes increased, George Emil Palade joined the booming field of cellular biology, and in the 1940s he turned his attention on yeast microsomes, small cellular bodies which were believed at the time to be nothing more than pieces of mitochondria. By using a transmission electron microscope (EM), George Palade was the first to show that the structure and chemical composition of the microsomes are in fact distinct from those of mitochondria. By 1956, Palade revealed microsomes to contain RNA and re-named them “ribosomes”. In 1975, for his overall work in cell biology, which comprised structural and functional studies of other cell organelles as well, Palade was awarded a share of the Nobel Prize in Physiology or Medicine.

In all organisms, the ribosome is the essential component of the translational machinery. Although slightly different in terms of the amount of their RNA and protein content (see Table I.1.), ribosomes from all kingdoms are composed of roughly 60 % ribosomal RNA (rRNA) and 40 % ribosomal proteins (r-proteins), distributed between two ribosomal subunits of unequal size.

Table I.1. The ribosome

Ribosome components		Eukaryotes (80S)		Prokaryotes (70S)		Mitochondria (55S)	
	rRNA		25-29S		23S		16-17S
Large subunit		60S	5S	50S	5S	39S	
			5.8S				
	proteins		49		33		48
Small subunit	rRNA	40S	16-18S	30S	16S	28S	12-13S
	proteins		33		21		29

The letter “S” stands for Svedberg units, which indicate how fast a particle sediments during ultracentrifugation, thus correlating with the size and weight of the particle. The Svedberg units are not additive, i.e. the combined sedimentation rates of the individual ribosomal subunits do not equal the sedimentation rate of the assembled ribosome.

The bacterial *Escherichia coli* (*E. coli*) 70S ribosome is formed by a large (50S) ribosomal subunit and a small (30S) ribosomal subunit (see Table I.1.). The 30S subunit contains a 1542 nucleotide rRNA strand (16S rRNA) and 21 different proteins numbered from S1 to S21, the letter “S” standing for “small”. The 50S subunit has two rRNA strands, a smaller 5S rRNA comprised of 120 nucleotides and a larger, 23S rRNA, with 2904 nucleotides. The large, 50S, subunit contains 33 proteins numbered from L1 to L25 and from L27 to L34, “L” standing for “large”.

All r-proteins are distinct independent proteins, with the following exceptions: (1) one protein is found in both subunits (S20/L26), (2) L7 and L12 are acetylated and methylated forms of the same protein (therefore named L7/L12), (3) L8 is a complex of L7/L12 and L10, and (4) L31 is known to exist in two forms, full length (7.9 kDa) and fragmented (7.0 kDa). Except for S1, which

has a molecular weight of 61.2 kDa, the other r-proteins have molecular weights ranging between 4.4 kDa (L34) and 29.7 (L2) kDa [1].

The *E. coli* ribosome's dimensions are approximately 170 x 230 x 250 Å [2], as determined by X-ray diffraction, and its molecular weight is 2.298 MDa. In order to ensure the correct function of such complex ribonucleoprotein machinery, the production and assembly processes of the ribosomal subunit components must be extremely efficient. All three *E. coli* 70S rRNA strands are transcribed as a single transcript from the rRNA operon in a cell growth rate dependent manner (reviewed in [3]). Co- and/or post-transcriptionally, the rRNA strands are processed by RNases (III, E, G, P, T), modified at approximately 30-40 specific positions by base methylation and/or pseudouridylation, partially folded and partially assembled with r-proteins (reviewed in [4-6]). In parallel with these rRNA transformations, the r-proteins are transcribed in a highly regulated manner, with more than 10 of them being post-translationally modified by methylation, acetylation and glutamylation (reviewed in [4, 7]). Although the role and order of many of these modifications is not yet fully understood, they have been proposed to enhance the stabilization of the secondary rRNA structure, or of specific rRNA-protein interactions, or to mediate various interactions between the ribosome and specific translation factors. Also, it has been proposed that these apparently unnecessary modifications of the rRNA and of the r-proteins may in fact serve as checkpoints throughout the process of ribosome assembly. Indeed, during further rRNA and r-protein assembly into rRNA tertiary structure, an extensive plethora of maturation factors, GTPases, helicases and other assembly proteins, specifically interact with the r-proteins and the rRNA to coordinate, guide and proofread the ribosomal assembly process.

The overall process of ribosome assembly is however not a strictly ordered succession of steps. The processes described above may happen in a dynamically coordinated order, designed to adapt to a variety of intracellular conditions, and to employ a diverse number of pathways, which, eventually, all lead to the formation of the complete 70S ribosome [8].

Once assembled, the two ribosomal subunits exist separately before and after translation, and only become associated during protein synthesis. Their association and dissociation depends on the concentration of Mg^{2+} , which *in vivo* is approximately 6 mM [9]. Ribosomal subunit association leads to the formation of the functional ribosome, which can exist (1) free in the cytoplasm and synthesizing cellular proteins (prokaryotic and eukaryotic ribosomes), (2) attached to the endoplasmic reticulum and producing mostly export proteins (eukaryotic ribosomes) or (3) within the mitochondria (mitochondrial ribosomes). Regardless of their cellular compartmentalization, ribosomes involved in active translation form clusters (called polyribosomes), i.e. several ribosomes attach one after the other to the same mRNA strand.

Initial negative staining EM studies [10] identified distinctive structural landmarks of each of the ribosomal subunits, which were later confirmed by low resolution cryo-EM maps [11]. The presently known high resolution structure of the bacterial ribosome is diagrammed in Fig. I.1.

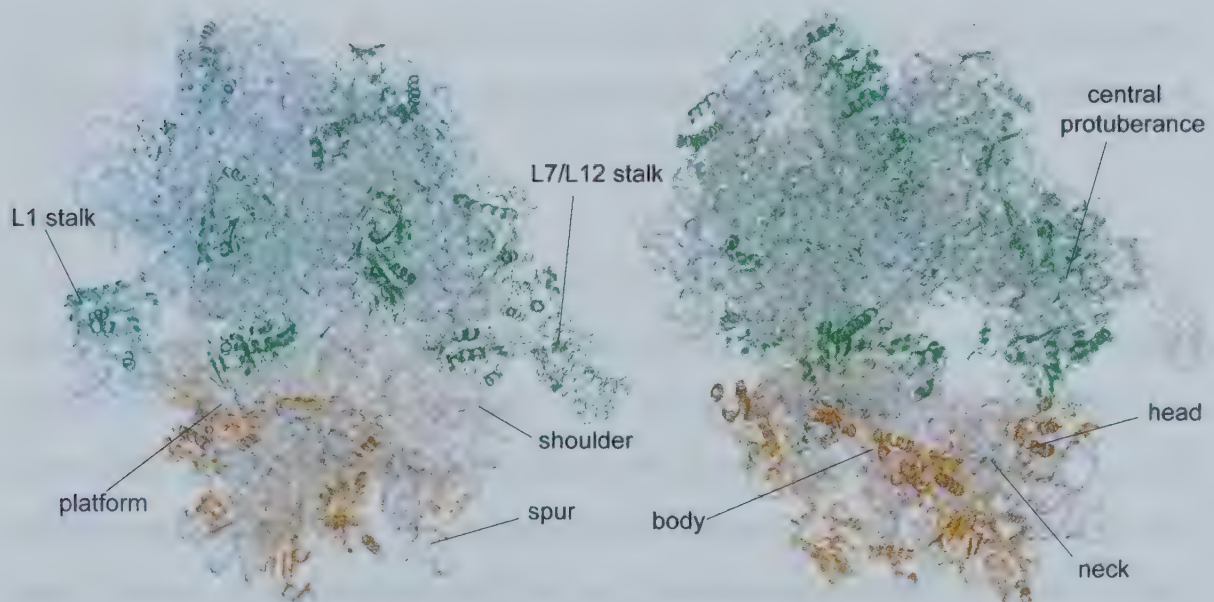


Fig. I.1. The macromolecular structure of the bacterial ribosome

The most recent crystal structure of the ribosome (PDB files 2WRI and 2WRJ) [12] was used to delineate the composition of the two ribosomal subunits. The rRNA and proteins of the 50S subunit are shown in light and dark green, respectively, while the 30S rRNA and proteins are shown in light and bright orange, respectively. The rotation angle between the two images of the ribosome is approximately 90°.

Higher resolution cryo-EM studies followed in the late 1990s ([13-14], 15 and 7.5 Å resolution respectively), and in the early 2000s, the first atomic resolution structures of the ribosomal subunits were determined [15-17] (2.4, 3.3 and 3.0 Å, respectively). These structural studies were awarded the Nobel Prize in Chemistry in 2009 and some of their coordinates were used to reconstruct the entire *Thermus thermophilus* (*T. thermophilus*) ribosomal particle at 5.5 Å resolution [18]. The *E. coli* vacant ribosome structure at 3.5 Å resolution followed shortly after [19], as did a cryo-EM based structure (11-15 Å resolution) of an *E. coli* ribosome involved in the ongoing synthesis of a polypeptide chain [20].

From this point on, the ribosome has been visualized in different functional states, complexed with deacylated tRNA and mRNA fragments [21-22] (3.7 and 2.8 Å, respectively), as well as with longer, more physiological mRNA molecules [23] (approximately 5 Å resolution). Recently, less than 4 Å resolution structures of the ribosome complexed with initiation [24], termination [25-27] and elongation [12, 28-29] factors have been revealed, as well as the structure of EF-G bound to the ribosome at various stages during elongation [12, 30].

Other than the ribosome and its specific translational protein co-factors, the protein synthesis machinery also requires the presence of mRNA and tRNAs.

I.1.2 The mRNA

The mRNA is a chemical "blueprint" of the genetic information encoded in the DNA template of the gene from which it is transcribed. Unlike most eukaryotic mRNAs, which code for only one protein (monocistronic mRNAs), most of the mRNAs found in bacteria and Archaea are polycistronic [31], i.e. one mRNA molecule carries the information of several genes, co-regulated in the same operon. Polycistronic mRNAs are subsequently translated into several proteins, which usually have related functions. Also, unlike eukaryotic mRNAs, which undergo an extensive process of post-transcriptional modification (i.e. intron removal, 5' cap addition, 3' polyadenylation) and transport before being translated by the ribosome, prokaryotic mRNAs do not need to be post-translationally spliced, modified or transported, and the ribosome attaches to the

mRNA and begins translation before transcription is completed, i.e. bacterial translation is co-transcriptional.

As in the DNA, the genetic information contained by the mRNA is encoded in triplet sequences of nucleotides, i.e. codons. Based on the possible permutations of the four RNA bases, there are 64 codons in the genetic code. This genetic code is degenerate [32]. Out of the 64 codons, 61 encode for the 20 essential amino acids (sense codons), with each amino acid being represented by up to six codons, while some codons signal two different translational functions. The remaining three codons are non-sense or stop codons (UAG, UAA and UGA), signaling translational termination. Two amino acids are encoded by only one codon (methionine and tryptophan), while the other amino acids are encoded by two or three codons. The codons encoding for the same amino acid differ only by the last nucleotide, except for the three amino acids encoded by six codons (leucine, serine and arginine), which also have differences in the first and second nucleotides. In addition, the same codon can signify two functions during translation. For example, UGA, in addition to signaling termination of protein synthesis, can also function as a sense codon and encode for the unusual amino acid selenocysteine in bacteria. Also, AUG encodes for methionine when encountered during the elongation phase of polypeptide chain synthesis, but can also function as a start codon and encode the formylated form of methionine, which delineates the initiation of protein synthesis. Both UGA and AUG codons exert their dual function in translation under additional regulatory mechanisms, such as specific secondary structure mRNA [33-34] and tRNA [35] elements.

The ribosome “reads” the mRNA from 5’ to 3’, which is primarily bound to the small subunit, with only eight nucleotides exposed to the inter-subunit interface [23, 36]. Approximately 30 nucleotides of the mRNA are wrapped in a groove which circles the neck of the small subunit, after which the mRNA follows first an upward path between the platform and the head, and then a downward path between the head and the body of the 30S. The 5’ UTR of the mRNA is stabilized on the ribosome by interactions between the Shine-Dalgarno (SD) sequence, formed of six bases of the mRNA (AGGAGG), and six complementary

bases of the 16S rRNA, called the anti-SD sequence, situated in between the platform and the head of the 30S subunit. Although the SD sequence plays a major role in the proper positioning of the mRNA on the 30S subunit and has been shown that mRNAs lacking the SD sequence have a more labile 5' end with respect to the 30S [23], the r-protein S1 as well as certain secondary structure characteristics of the mRNA have also been proposed to participate in stabilizing the binding of the mRNA to the small ribosomal subunit [37-38].

Although not as structured as the rRNA, the mRNA does have some secondary structure elements, which would prevent it both from making intimate contact with the 30S as described above, and from being translated in frame by the ribosome. Thus, similarly to the RNAP, the small ribosomal subunit has been shown to harbor intrinsic helicase activity, which is manifested during both initiation and elongation. Some of the platform proteins may participate in mRNA unwinding during initiation: S7, S2, S11, S18, S21 [39], as well as S1, which has been shown to harbor some helicase activity [40]. During elongation, as the mRNA is being advanced through a hydrophobic clamp formed by proteins S3, S4 and S5 in between the head and the shoulder regions of the 30S, S3 and S4 act as helicases to unwind the mRNA, thus maintaining in-frame translation [41].

In *E. coli*, the lifetime of mRNAs ranges from a few seconds to little over one hour. Since the mRNA amount is correlated with its corresponding encoded protein levels, mRNA turnover regulation can be a means of tuning the cell's needs to environmental stressors [42]. In *E. coli*, mRNA turnover is regulated by three major classes of molecules: ribonucleases (RNases), mRNA binding proteins, and small non-coding RNAs (reviewed in [43]).

I.1.3. The tRNAs

tRNAs bring the correct amino acid encoded by the mRNA to the ribosome during protein synthesis. Each tRNA is amino acid-specific, however, more than one species of tRNA may carry the same amino acid. Therefore there are more types of tRNA than amino acids. For example, mammalian cells have roughly 150 tRNAs, while *E. coli* has 49 different tRNA species.

E. coli has approximately 80 tRNA genes [44] which probably undergo similar regulatory mechanisms as the rRNA genes, i.e. by receiving negative feedback from products of rRNA operons [45]. Transcription of the tRNA genes results in a 120-130 nucleotides long “precursor” tRNA molecule, which is then processed to form the mature tRNA by (1) nucleolytic cleavage and trimming (done by RNases P, P2, Q and PIII), (2) nucleoside modification, achieved primarily by methylation, and by (3) selective 3’ terminal addition of nucleotides (CCA), for those tRNAs which do not gain a 5’-...CCA-3’ end following RNase Q cleavage.

In 1965, the first primary [46], and secondary, “cloverleaf” shaped [47], tRNA structures were determined. The “cloverleaf” contains double helical stem regions (nearing stretches of complementary bases) and loop domains (resulting either from lack of sequence complementarity or due to the presence of modified bases, which prevents base pairing). The 3’ end arm of the tRNA consists of seven base pairs and four unpaired nucleotides, out of which the last three are always 5’-...CCA-3’, and the other is A or G. The amino acid molecule attaches to the 3’ terminal A, which is why the 3’ end arm is called the acceptor stem, while the constant CCA sequence is known as the amino acid binding site. The other arms are termed the D arm, the anticodon arm (contains a trinucleotide sequence which pairs with its corresponding mRNA codon during translation), the variable arm, and the T ψ C arm. The 5’ end of tRNA is either G or C. tRNA tertiary structure results from further folding of the secondary structures, and is achieved by hydrogen bond formation between unpaired bases situated at distant sites, between bases and the ribose phosphate backbone, and between different backbone residues. The resulting compact structure resembles the letter “L”, with one limb formed by the acceptor and T ψ C stems and the other by the D arm and the anticodon stem.

In order to participate in translation, the tRNAs need to be charged with the corresponding amino acid encoded in the tRNA anticodon and in the mRNA codon. During translation, the codon anticodon pairing on the ribosome is however only complementary for the first two bases in the tRNA and mRNA,

while the third position can "wobble", i.e. is not necessarily base paired between the mRNA codon and the tRNA anticodon. Such flexibility in the genetic coding ultimately enables a single tRNA to base pair with more than one mRNA triplet codon sequence, which explains how more than one mRNA codon is allowed to encode for one amino acid.

Charging of the tRNAs with the correct amino acid is catalyzed by aminoacyl-tRNA (aa-tRNA) synthetases, which recognize certain "identity elements" of the tRNA, i.e. the acceptor stem and anticodon loop (for a detailed review, see [48]). tRNA charging is a two step, ATP-dependent, process. First, the amino acid is activated by reacting with ATP to form aa-AMP and PPi. This reaction is reversible in the presence of reaction products, however the enzyme pyrophosphatase readily hydrolyzes PPi upon its formation, thus driving the reaction uni-directionally. In step two, the 2' or 3' hydroxyl of the terminal adenosine of the tRNA attacks the amino acid carbonyl carbon atom, releasing AMP. The 2' and 3' isomers are spontaneously interchangeable in solution and the exact position of the attached aminoacyl group has been shown to be functionally irrelevant.

Based on the tRNA identity elements they recognize, on the overall fold of their ATP binding domain, and on the isomeric position of the 3' adenosine to which they attach the aminoacyl moiety, the aa-tRNA synthetases have been classified in two distinct classes. Class I enzymes have a Rossmann fold (two three sets of alternating α and β strands) of the ATP binding domain, yield the 2' isomer and recognize both tRNA identity elements, while class II synthetases have an anti-parallel β sheet arrangement of the ATP binding domain, create a 3' isomer and recognize only the tRNA acceptor stem.

In order to maintain the fidelity of translation, tRNA charging must be highly specific and tightly regulated. Each aa-tRNA synthetase is amino acid specific (there are 20 synthetases, one for each amino acid) and upon tRNA recognition, the complex undergoes a cognate specific conformational change. Furthermore, non-cognate charging of tRNAs is subjected to a proofreading

hydrolysis mechanism, in which the bond between the aminoacyl moiety and the tRNA is cleaved by water attack.

After charging, some amino acids are pre-translationally modified. For example, the Met-tRNA^{Met} is formylated in the presence of tetrahydrofolate by methionyl transformilase (MTF), yielding the initiator fMet-tRNA^{fMet}. Similarly, in presence of selenophosphate, Ser-tRNA^{Ser} is converted to selenocysteine-tRNA^{Ser} by selenocysteine synthetase, while Asp-tRNA^{Asn} and Glut-tRNA^{Gln} are converted to Asn-tRNA^{Asn} and Gln-tRNA^{Gln}, respectively, by amidotransferases.

The tRNAs bind to the mRNA ribosome complex in a Mg²⁺-dependent manner [49], to three pre-determined binding sites situated inside the inter-subunit cavity and which span both ribosomal subunits: A, P and E sites. The aa-tRNA binds to the A site, the peptidyl-tRNA sits in the P site, while the deacylated tRNA occupies the E site. While bound to the ribosome, the tRNAs interact mostly with rRNA [18, 50-52] and with only a few r-proteins (S12, S13 and L16 in the A site, S9, S13, L5 and L27 in the P site and S7, L1 and L33 in the E site).

Unlike mRNAs, stable RNAs like rRNAs and tRNAs are degraded only under certain stress conditions, including nutrient starvation, or when defective (reviewed in [53]). tRNA degradation is initiated by endonucleolytic cleavages, followed by exoribonuclease cleavage. RNA fragments are further polyadenylated and cleaved by the degradosome and/or RNase E. Defective tRNAs are usually degraded at the tRNA precursor stage, by polyadenylation, followed by PNPase, and probably also by RNase R, cleavage.

1.2. BACTERIAL TRANSLATION

Translation is the process linking genotype and phenotype, by using the RNA product of transcription to direct protein synthesis. Translation is carried out by the ribosome, in combination with RNA and protein co-factors, altogether forming the translational macromolecular machinery responsible for polypeptide chain synthesis. Although the ribosomal subunits function as a whole during translation, some translational functions are primarily attributed to the small

ribosomal subunit, such as mRNA binding and decoding, while the large ribosomal subunit is primarily associated with peptide bond formation.

Translation can be viewed as a cyclical process, with three distinct phases: initiation, elongation and termination. Each of these processes is associated with the participation of specific protein factors, named after the translational phase in which they function: initiation factors (IFs), elongation factors (EFs) and release factors (RFs), also termed termination factors.

I.2.1. Initiation

Bacterial translation initiation (reviewed in [54], see Fig. I.2.) involves three protein factors (IF-1, IF-2 and IF-3), which coordinate ribosomal subunit association in the presence of the initiator fMet-tRNA^{fMet}, base paired in the P site with the AUG mRNA codon. At the end of initiation, the A and E sites are empty.

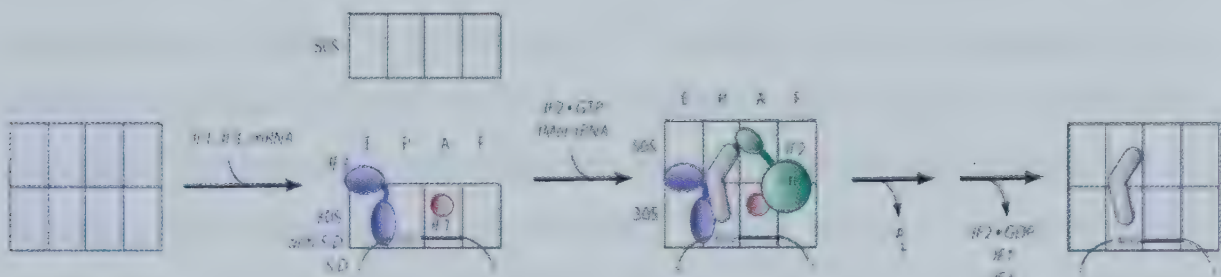


Fig. I.2. Bacterial translation initiation

The two ribosomal subunits are shown as superimposing rectangles, the initiator tRNA is gray, and the mRNA, with its SD sequence, is shown in black. The ribosomal tRNA binding sites, mRNA and IFs are as marked.

IF-1 is a small 8.2 kDa (*E. coli*) protein, which upon binding to the A site of the small ribosomal subunit, flips out A1492 and A1493, the two major helix 44 bases needed for A site decoding during elongation. By binding over these nucleotides, IF-1 in fact blocks A site binding of any aa-tRNAs during initiation. Also, IF-1 may promote further, more extensive conformational changes of the 16S rRNA, which overall prevent ribosomal subunit association [55]. IF-1 also stimulates the activity of IF-2 and IF-3, and stabilizes IF-2 binding. **IF-2** is a 97.3 kDa (*E. coli*) GTPase which associates with the initiator fMet-tRNA^{fMet} and

favors its placement into the P site. GTP hydrolysis by IF-2 triggers the release of the initiator tRNA into the P site and the dissociation of the IFs from the ribosome, which signals the beginning of elongation. **IF-3** is a 20.3 kDa (*E. coli*) double domain protein, which places its NTD into the 30S E site, while its CTD makes contact with the anticodon loop of the initiator tRNA. Its dual interactions serve to block the ribosomal E site, help stabilize the fMet-tRNA^{fMet} and the AUG mRNA codon into the P site and prevent ribosomal subunit association. Also, IF-3 shows proofreading activity against unspecific tRNAs, and favors subunit dissociation if non-initiator tRNAs are recruited to the P site, or if no AUG codon is found in frame on the mRNA.

In *E. coli*, AUG is used as a start codon in 83 % of cases. Although other start codons can be also used (14 % GUG, 3 % UUG and less than 1 % AUU and CUG), fMet-tRNA^{fMet} is always the initiator tRNA. AUG can also code for Met-tRNA^{Met}, if encountered during elongation. The following structural particularities, specific to fMet-tRNA^{fMet}, enable it to be recognized as an initiator rather than an elongator tRNA: (1) the formyl group, which is specifically recognized by IF-2, (2) nucleotides C1 and A72 are not base paired, which facilitates the positioning of fMet-tRNA^{fMet} CCA acceptor arm within the catalytic site of MTF, (3) the A11-U24 base pair presence in the D-arm, which is specifically recognized by MTF; (4) the conserved three successive G-C base pairs in the anticodon arm, that confer a specific structure to this region, which in turn allows it to be specifically identified by IF-3 for P site selection. Interestingly, a recent 3.1 Å resolution crystal structure of *E. coli* fMet-tRNA^{fMet} revealed “extended” (four bases rather than three) codon anticodon interactions with the mRNA, as well as more stable stacking in the anticodon region, coupled with more extensive interactions with the 16S rRNA [56]. These structural interactions are specific to the initiation phase and probably stabilize P site accommodation of the fMet-tRNA^{fMet}.

mRNA binding to the small ribosomal subunit during initiation is believed to be a two step process. First, the structured, unfolded mRNA is transiently docked onto the platform of the 30S subunit, with the participation of the SD or of

other secondary structure elements of the mRNA situated upstream from the SD. At this point, there is no codon-anticodon pairing interaction between the AUG and the initiator tRNA, and the resulting complex is inactive and unstable. The second step is mediated by the 30S itself in cooperation with IFs and with fMet-tRNA^{fMet}, and involves mRNA accommodation into the 30S mRNA channel, as described in 1.1.2. Upon mRNA unfolding and correct accommodation, the anticodon arm of the initiator tRNA base pairs with the initiator mRNA codon. This interaction stabilizes and activates the pre-initiation complex.

With the exception of IF-3, which is believed to bind to 30S as early as the termination phase of translation, all other components of the pre-initiation complex (30S, mRNA, IF-1, IF-2·GTP and fMet-tRNA^{fMet}) associate with each other in a cooperative and synergistic manner. After initiation complex activation, IF-2 hydrolyzes GTP, which triggers the dissociation of all IFs, promotes 50S association and prepares the 70S ribosome for elongation [57].

Compared with bacteria, eukaryotic initiation (reviewed in [58]) is completed by approximately 12 core eIFs (eukaryotic initiation factors) and five auxiliary factors. The higher number of co-factors than in bacteria is primarily due to the complexity of mRNA recognition processes and of initiation complex formation mechanisms. Also, these factors form multi protein complexes (eIF-4 is a complex of eIF4-A2, eIF4-A3, eIF4-B, eIF4-E, and eIF4-G) and bear more specialized functions than bacterial IFs (for example, eIF-4A is an ATPase with mRNA helicase properties, similar to the bacterial 30S subunit). Still, some eIFs share functional similarities with the bacterial IFs (eIF-1 and eIF-1A with IF-1, eIF-2 with IF-2, and eIF-3 with IF-3).

The eIF-4 complex recognizes, unwinds and anchors the mRNA to the pre-initiation complex, which comprises the 40S subunit, eIF-3 (E site), eIF-1 and eIF-1A (A site), as well as the eIF-2·GTP·Met-tRNA^{Met} ternary complex (P site). This complex may also include eIF-5A. eIF-5A is structurally similar to the bacterial protein EF-P (Elongation Factor P) [59], which helps stabilize the fMet-tRNA^{fMet} in the P site [28], thus favoring the formation of the first peptide bond. Both factors are discussed in detail in chapter IV.

As eukaryotic mRNAs lack the SD sequence, the pre-initiation ribosomal complex scans the mRNA from 5' to 3' until the AUG initiation codon reaches the small ribosomal subunit's P site. Upon initiation codon base pairing with the anticodon loop of the initiator tRNA, eIF-5A activates GTP hydrolysis by eIF-2, which leads to the dissociation of all eIFs from the ribosome.

Large ribosomal subunit association to the remaining mRNA·Met-tRNA^{Met}·40S complex is catalyzed by the ribosome dependent GTPase eIF-5B.

I.2.2. Elongation

Elongation undergoes similar steps in prokaryotes and eukaryotes. Bacteria have three EF's, Elongation Factor Tu (EF-Tu), Elongation Factor Ts (EF-Ts) and Elongation Factor G (EF-G). Their eukaryotic counterparts are EF-1A, EF-1B and EF-2, respectively.

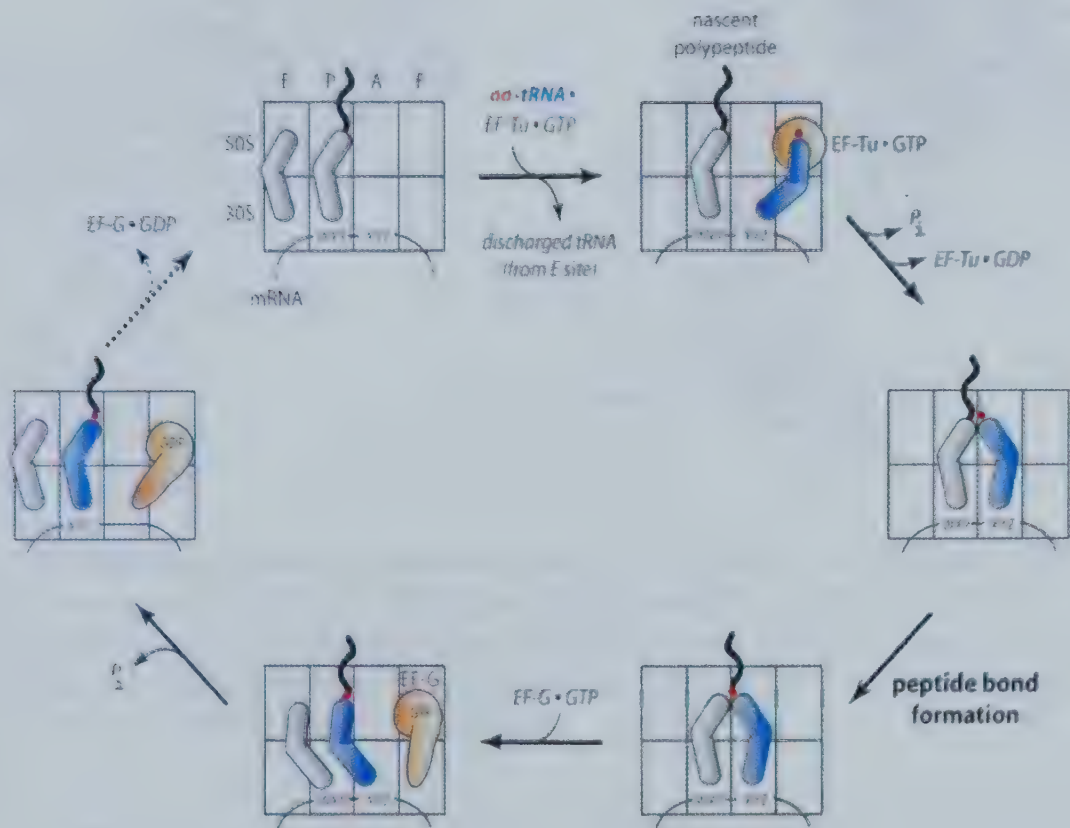


Fig. I.3. Bacterial translation elongation cycle

The ribosome, tRNAs and mRNA are represented as in Fig. I.2. The amino acid attached to the tRNA is shown as a red dot, while the growing polypeptide chain is shown in black. EF-G and EF-Tu are as marked.

EF-Tu and EF-G are GTPases, while EF-Ts is a GTPase exchange factor (GEF) for EF-Tu. EF-Tu delivers the charged tRNAs to the ribosome, while EF-G translocates the two tRNAs and mRNA after peptide bond formation has taken place on the ribosome.

Although these two GTPases act at different steps in elongation and have different functions, they share certain structural similarities: domains I and II of EF-Tu and EF-G are similar, whereas domains III, IV and V of EF-G resemble the aa-tRNA bound to the active (GTP) form of EF-Tu [60-61] (Fig. I.4.).

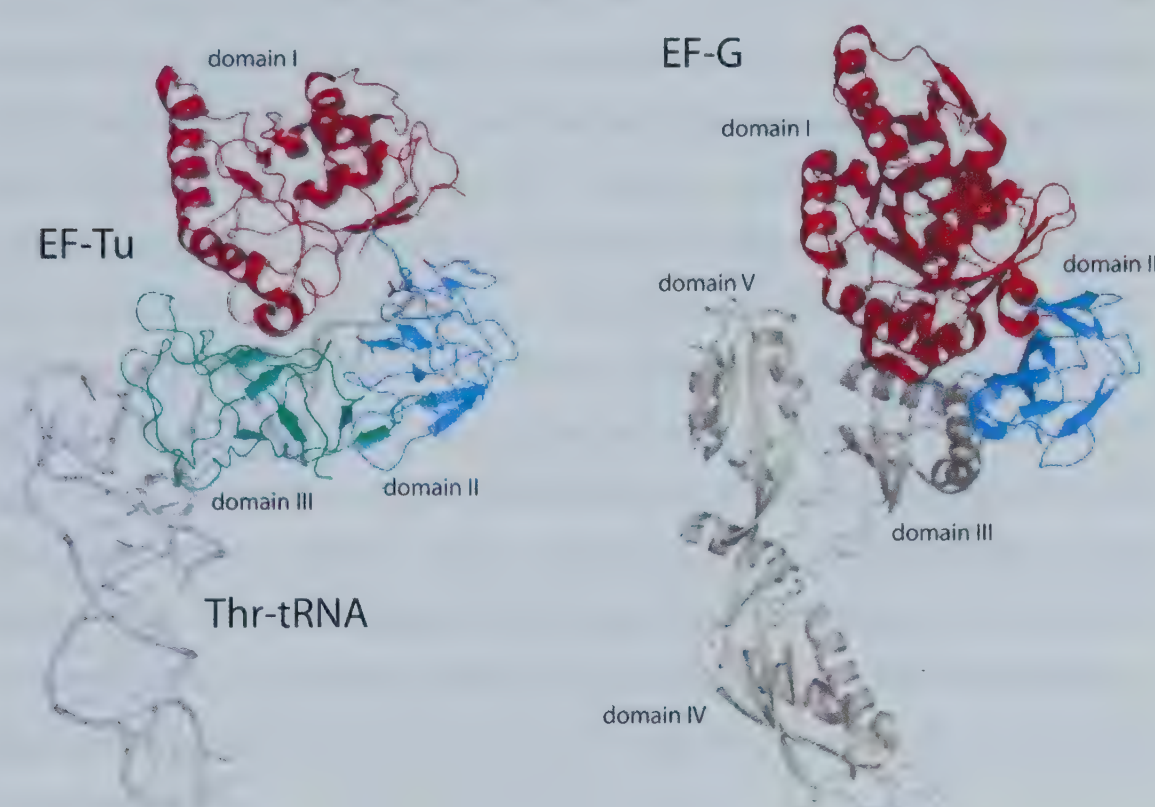


Fig. I.4. Structural mimicry between the EF-Tu ternary complex and EF-G
 EF-Tu bound to Thr-tRNA^{Thr} (PDB file 1EFT), and EF-G (PDB file 1WDT), are shown side by side. The corresponding domains of both EF's are: domains I in red and II in cyan. EF-Tu domain III is green. The tRNA (Thr - black) and domains III, IV and V of EF-G are shown in wheat.

Functionally, EF-Tu and EF-G associate cyclically, alternatively and cooperatively with the ribosome [62-64] as fast as up to 20 times per second *in vivo* [65]. They bind to identical sites on the ribosome [66], which acts as a GTPase activating factor (GAP) for both proteins [67-69]. Also, the ribosomal binding sites of the corresponding domains overlap [70]. The common ribosomal

binding site for EF-Tu and EF-G is called the F (factor) site. The F site is located in the large ribosomal subunit and is composed of two elements: the sarcin-ricin loop (SRL) and the GTPase activating center (GAC). The SRL is a universally conserved region in domain VI (helix 95) of the 23S rRNA, targeted by the α -sarcin and ricin toxins, which contacts elongation factors in the vicinity of their GTP-binding site. The GAC contains helices 42, 43 and 44 of the 23S rRNA, as well as L11 and the L7/L12 stalk.

The EF's discrimination mechanism between post- and pre-translocational ribosomal functional states has been described in detail in [71]. The GAC has been visualized both away ("open") or close ("closed") to the loop-end of the helix 89, which translates into the post-translocational ribosome state (with EF-Tu in the GTP state and EF-G in the GDP state), or into the pre-translocational ribosomal complex (which has EF-Tu in the GDP state and EF-G in the GTP state), respectively. The GAC interacts with domains I of both EF-Tu and EF-G, which have a GTPase specific type of folding, hence their alternative names of "G domains". The G domain of EF-G contains an additional insertion, termed the G' domain, which is specific to EF-G. Although the GAC interacts similarly with the G domain of EF-Tu and with the G' domain of EF-G, the conformations and locations of the GTP binding sites of EF-Tu and EF-G are different, which results in different orientations of the mobile GAC element relative to the immobile SRL. These alternative conformations of the GAC may dictate which EF will bind to the ribosome.

EF-Tu (for detailed reviews, see [72-73]) comes into play either after initiation complex formation (at the beginning of elongation) or after EF-G translocation (during elongation), when the P site is occupied by either fMet-tRNA^{fMet} or by peptidyl-tRNA, respectively, and the ribosomal A site is vacant. In its GTP (active) state, EF-Tu forms a ternary complex with aa-tRNA by trapping the aminoacylated acceptor arm of the tRNA in its inter-domain core (Fig. I.2.). The EF-Tu·GTP·aa-tRNA ternary complex is recruited to the ribosome by interactions with the r-protein L7/L12 [74]. At this point, the ternary complex is

held in place by EF-Tu interactions with the 50S in the F site, which places the aa-tRNA in the 30S A site in a codon-independent manner.

The next step is called “decoding” and refers to monitoring of correct base pairing between the incoming aa-tRNA and the existing A site mRNA codon by the 30S subunit. Three nucleotides of the 16S rRNA undergo conformational rearrangements and engage in interactions with the three codon anticodon base pairs. A1492 and A1493 of the 16S rRNA form hydrogen bonds with the first two base pairs, while G530 helps monitor the second base pair. The third base pair is not monitored by the 30S subunit, which explains the possibility of “wobble” base pairing at this position [75]. This triad of interactions (30S-tRNA-mRNA) pulls on the tRNA and “kinks” it so that its anticodon domain is slightly displaced to reach into the decoding center, while its acceptor end is still immobilized onto EF-Tu. The displacement of the anticodon domain leads to a global twisting of the tRNA molecule, which in turn affects the interactions of the aa-tRNA acceptor arm with the G domain of EF-Tu, and may be involved in triggering GTP hydrolysis by EF-Tu. Aside from the localized A site 16S rRNA changes, the small ribosomal subunit also undergoes more extensive (global) conformational rearrangements, which, in the end enable the “closed” 30S conformation required for aa-tRNA selection by the ribosome [76].

Cooperatively, tRNA twisting, 30S conformational rearrangements and 50S GAC opening to signal a post-translocational complex, activate GTP hydrolysis by EF-Tu. Although the detailed chemistry of this mechanism [77] is not yet fully understood, theoretically, the γ phosphate of GTP, stabilized by R59, would be attacked by a water molecule, which is stabilized and/or activated by H85. Residues I61 and V20 may form a hydrophobic gate, which opens up to facilitate water attack (Fig. I.5.; the residues used to exemplify the EF-Tu GTP hydrolysis mechanism are *T. thermophilus* numbering). GTP hydrolysis by EF-Tu unlocks extensive conformational changes of two conserved “switch” regions (termed switch I and switch II) within the G domain of EF-Tu, which cooperatively push domain II down and away from the inter-domain core of EF-Tu (Fig. I.5.), thus releasing the aa-tRNA in the A site.

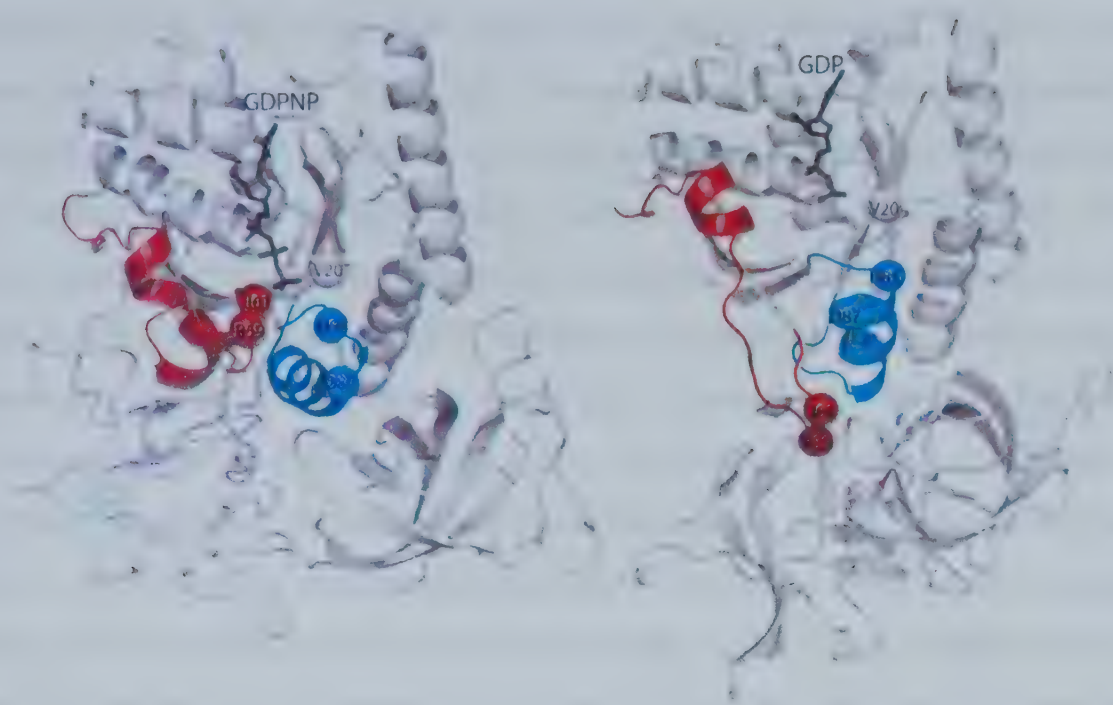


Fig. I.5. Conformational changes of EF-Tu induced by GTP hydrolysis

EF-Tu is shown comparatively in the GTP state, left (PDB file 1EFT) and in the GDP state, right (PDB file 1TUI). The bound nucleotides are colored black. Switch I is shown in red, switch II is shown in cyan, and the residues involved in GTP hydrolysis are shown as spheres centered on the C_{α} atoms.

Once the tRNA acceptor arm is released from EF-Tu's grip, the tRNA undergoes a process called accommodation, which entails a complex rotation and translation movement of its molecule, during which the acceptor arm is pulled over 70 Å distance into the 50S A site, while the anticodon stem remains anchored in the 30S A site through its interactions with the mRNA and the 16S.

In parallel, EF-Tu dissociates from the ribosome in complex with GDP. Since EF-Tu has a higher affinity for GDP than for GTP, it requires EF-Ts to facilitate GDP release. EF-Ts binds to the EF-Tu·GDP complex, removes GDP, and facilitates EF-Tu recycling, i.e. the formation of the ternary complex, under conditions of sufficient concentrations of GTP and aa-tRNAs.

The overall error rate of translation *in vivo* is believed to be on the order of 10^{-3} - 10^{-4} [78]. Although incorrect tRNA charging and frame shifting can also account for translational inaccuracy, incorrect tRNA selection by the ribosome is the major source of translational errors and undergoes multiple proofreading mechanisms. Firstly, decoding is the step at which most non-cognate tRNAs are

rejected: if the 30S A site 16S rRNA bases detect “wobbling” at any of the positions 1 and 2, the tRNA and ribosomal conformational changes which normally trigger GTP hydrolysis by EF-Tu do not take place, and the entire EF-Tu ternary complex is rejected from the ribosome [76, 79]. Secondly, GTP hydrolysis by EF-Tu can be inhibited in the presence of defective or near-cognate tRNAs [79], which probably fail to transmit the appropriate tRNA conformational bending to the tRNA acceptor arm, and to the G domain of EF-Tu [80]. Thirdly, if GTP hydrolysis on EF-Tu is triggered in the presence of non/near-cognate or defective aa-tRNAs, the GTP hydrolysis kinetics are slower than for cognate tRNAs [81]. Fourth, any near-cognate which bypasses previous proofreading steps, will not accommodate correctly upon release from EF-Tu, which renders it unstably bound to the ribosomal A site, and, in turn, rejected from the ribosomal complex [81].

After correct A site placement of the in-coming aa-tRNA, rapid peptide bond formation (over 300 s^{-1} and over 10^7 faster on the ribosome than in solution) is catalyzed by the ribosomal PTC (reviewed in [82]). The PTC is a symmetrical rRNA pocket situated deep into the core of domain V of the 23S rRNA. The PTC has three structural components: two “walls”, formed of rRNA, which contact the acceptor arms of the A and P site tRNAs, and are termed the A and P loops, respectively, and a “ceiling”, that looks into the polypeptide “exit” channel, which accommodates the growing polypeptide chain attached to the peptidyl-tRNA. In *E. coli*, the CCA end of the A site tRNA interacts with A loop bases U2555, G2553, U2556 and G2583, while the 3' CCA end of the P site tRNA interacts with P loop bases G2251, G2252, A2451 and A2450. The acceptor arms of the A and P site tRNAs are related by 180° rotation (mirror image symmetry), which facilitates peptide bond formation and transfer of the P site polypeptide chain to the A site aa-tRNA.

Proteins are formed by the ribosome starting from their N terminus. The α -amino group of the A site aa-tRNA attacks the carbonyl carbon of the P site peptidyl-tRNA. Peptide bond formation proceeds through a tetrahedral oxyanion intermediate and involves the aminolysis of the acyl-ester link between the

carbonyl carbon of the peptidyl moiety and the O3' atom of the A76 P site tRNA.

Structural studies revealed that there are no r-proteins situated within 18 Å from the PTC, thus revealing the ribosome to be a ribozyme [83]. This study proposed one of the first mechanisms for ribosome catalyzed peptide bond formation: the tetrahedral oxyanion intermediate forms a hydrogen bond with the N3 atom of A2451 of 23S rRNA, which supposedly played two roles: that of a general base and that of an oxyanion hole. However, mutagenesis and DMS modification studies of A2451 revealed no change in the ribosome's catalytic activity, plus increasing experimental evidence seemed to indicate that none of the other conserved 23S rRNA residues situated within the PTC are involved in catalysis. Subsequent studies revealed that the rate of peptide bond formation can be inhibited up to 10^6 fold on the ribosome and up to 10^2 fold in solution if the A76's O2' hydroxyl group of the P site tRNA was removed or replaced with a fluoro group. Further studies showed that the O2' hydroxyl group was however not important for catalysis *per se*, but that it is critical for proton shuttling between the attacking α -amino group and the leaving O3' hydroxyl.

To date, the accepted mechanism of peptide bond formation has three components: an entropic component, a general acid-base component and an electrostatic shielding component [82]. Upon binding to the ribosomal A site, the aa-tRNA induces conformational changes of the PTC, which result in proper orientation of reactive groups of both tRNAs and rRNA (entropic component). Peptide bond formation is chemically facilitated by the O2' hydroxyl group of A76 of the P site tRNA, which functions as a proton shuttle (general acid-base component), while the oxyanion hole stabilizing the tetrahedral intermediate is represented by a water molecule coordinated by the ribosomal bases A2602 and U2584 of the 23S rRNA (electrostatic shielding component).

The resulting polypeptide chain enters the "exit" channel situated in the ceiling of the PTC. This tunnel is formed primarily of rRNA, with only a few r-proteins, and travels almost 100 Å through the thickness of the 50S subunit. The polypeptide channel is narrow enough (in average 15 Å) not to allow for secondary protein structure formation (except perhaps for α -helices), plus r-

proteins L4 and L22 protrude inside the lumen at the narrowest point, probably acting as a folding control gate [83]. While polypeptide chain is ongoing at the PTC, chaperones (50S has been proposed to be one of them [83]) stationed at the exit gate start protein folding processes. In parallel, protein modification enzymes also come into play, such as peptide deformylase (removes the formyl group from the initial amino acid, fMet) and methionine aminopeptidase (removes the deformylated first Met residue).

After peptide bond formation, as the polypeptide chain advances through the exit tunnel, the A site tRNA now carries the peptidyl chain, while the P site tRNA is deacylated. The two tRNAs and the mRNA must now advance through the ribosomal cavity to clear the A site and make room for the next aa-tRNA encoded in the mRNA. The coordinated movement of tRNAs and mRNA from the ribosomal A/A and P/P sites to the P/P and E/E sites, respectively, is called “translocation”. The translocation machinery acts specifically on the tRNAs, while the mRNA may be only passively dragged along by its interactions with the tRNAs [84]. Although the ribosome alone can slowly mediate this process [85], efficient translocation is achieved only in the presence of EF-G, which may act to lower the ribosome’s kinetic barrier for translocation. In the absence of EF-G, the r-proteins S12 and S13 are believed to be responsible for preventing spontaneous translocation, probably by stabilizing the interactions of the aa-tRNA and the peptidyl-tRNA with the ribosomal A and P sites, respectively [86].

EF-G was discovered and purified in 1966, together with EF-Tu [63]. Initial crystallographic studies [87-88] showed a penta-domain GTPase (Fig. I.6., A.). Domain I has a typical GTP binding protein type of folding (hence its alternative name, the “G domain”) and an atypical intra-domain insertion (the G’ domain), which is not found in EF-Tu. The role of the G’ domain remains to be elucidated; however, it may cooperate in EF-G recruitment to the ribosome by L7/L12 [69, 74, 89] and in positioning of the G domain with respect to the GAC [90], such that the ribosome is able to discriminate between EF-Tu and EF-G in the F site. Domain III has been shown to be essential for ribosome activated GTP hydrolysis [91], while domains IV and V are essential for translocation [92].

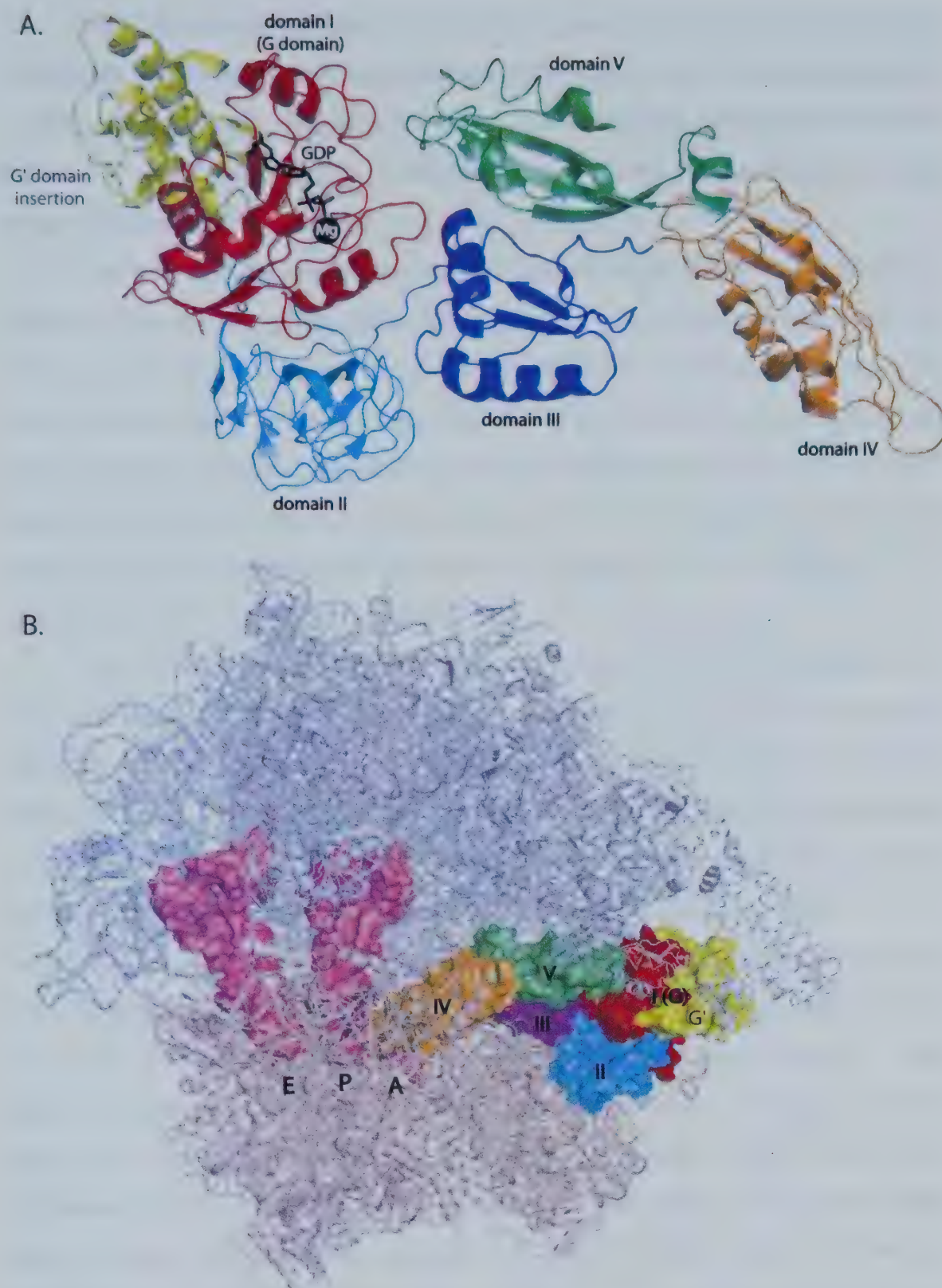


Fig. I.6. EF-G (PDB files 2WRI and 2WRJ)

A. EF-G cartoon: domain I is red (with G' - yellow), II is cyan, III is purple, IV is orange, and V is green. GDP and the Mg^{2+} ion are colored black.

B. EF-G bound to the ribosome: mRNA and tRNAs (hot pink) are shown in complex with EF-G and the ribosome (30S - light pink, 50S - light blue). EF-G, the two tRNAs and the mRNA are shown in Pymol surface mode.

Early functional [93] and structural [90, 94-95] studies, corroborated by recent high resolution crystallographic data [12], placed EF-G on the ribosome at the inter-subunit cavity. Domains I and V of EF-G interact with the 50S subunit, domains II and III face the 30S, while the body of domain IV interacts with both ribosomal subunits (Fig. I.6., B.).

The role and the mechanism of GTP hydrolysis by EF-G on the ribosome has long been an intriguing piece of the translational puzzle. Assuming EF-G behaves as a typical GTPase (active in its GTP form and inactive in its GDP form), it was initially proposed that EF-G binds to the ribosome and hydrolyzes GTP to catalyze translocation, followed by the dissociation of its inactive, GDP form [96]. However, detailed kinetic analysis of the EF-G catalytic cycle showed that GTP hydrolysis happens immediately following EF-G binding to the ribosome and before translocation [97].

The mechanism of GTP hydrolysis was initially believed to be identical in EF-G and EF-Tu, based on their structural similarities, however it has been shown that their switch I regions are not interchangeable [98] and that switch I of EF-G may move in the opposite direction from switch I of EF-Tu upon GTP hydrolysis [99]. Also, unlike EF-Tu, EF-G binds to the ribosome with either GDP or GTP [97, 100], and it has similar affinities for GTP and GDP in solution [101].

To date, the GTP hydrolysis mechanism for both factors remains unclear. It has been suggested that EF-G and EF-Tu use arginine fingers to catalyze GTP hydrolysis, by analogy with other GTPases, such as Ras. However, well conserved arginines within the G domain situated in close proximity with the bound GTP nucleotide, have not been shown to be essential for GTP hydrolysis activation in either EF-Tu or EF-G [102-103]. Further search for arginine finger donors among the r-proteins, especially L7/L12 [104], yielded no results. Interestingly, a remote arginine residue of EF-G, R29, which is not present in EF-Tu, turned out to be essential for GTP hydrolysis in EF-G [105]. Still, this residue is about 11 Å away from the GTPase center of EF-G, thus the G domain would have to undergo significant rearrangements to position R29 close to the GTP γ phosphate.

In EF-G, as shown in Fig. I.7. (residues are *T. thermophilus* numbering), the G domain switch regions, and especially switch I, undergo significant conformational changes between the GTP and the GDP. In the GTP bound state [30], the γ phosphate of GTP is stabilized by a hydrophobic core formed between switch I (E54 and H58), switch II (Y84 and F87) and domain III residues. Switch I helps position the Mg^{2+} ion through interactions with D50 and T61, while K15, a conserved P loop (phosphate binding) lysine shown to be important for EF-G binding and GTP hydrolysis, is positioned to interact with both the β and γ phosphate of GTP.

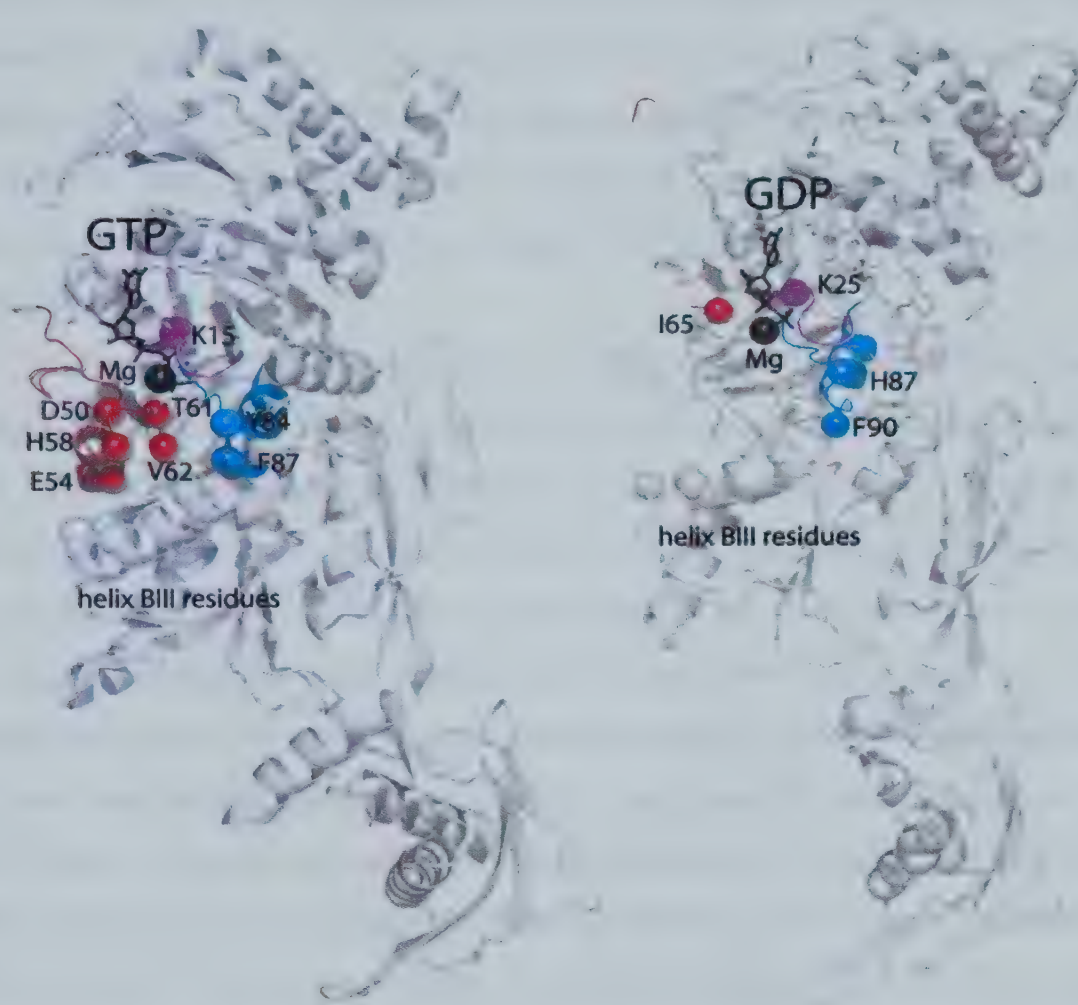


Fig. I.7. The EF-G switch regions and residues important for GTP hydrolysis EF-G, in the GTP (PDB file 1WDT) and GDP (PDB file 1WRI) states, is shown in white, with switch I colored red, switch II - cyan and the P loop - magenta. GTP, GDP and the Mg^{2+} ion are shown in black. Individual residues considered to be important for the GTPase catalysis mechanisms are shown as spheres centered on the C_{α} atom. The numbering of residues is *T. thermophilus* EF-G-2 for the GTP state and EF-G-1 for the GDP state.

Following GTP hydrolysis, switch I becomes disordered and moves away from helix B_{III} residues [99], destabilizing the hydrophobic GTP binding core [12], while the P loop lysine side chain is pointing towards switch II [87-88, 106-109]. Still, these conformational changes are not sufficient to trigger GTP hydrolysis on EF-G. It has been proposed that interactions between domain I of EF-G and L7/L12 during its recruitment to the ribosome may initiate the required conformational changes of the G domain, thereby inducing a so-called “GTPase transition state” [68-69, 89]. Cryo-EM reconstructions of the EF-G-2 crystal structure bound to the ribosome revealed that switch I interacts with helix 14 of the 16S rRNA in the 30S subunit, switch II is in contact with the SRL, while domain III (also shown to be essential for GTP hydrolysis [91]) interacts with r-protein S12 [30]. Together, these studies suggest that further interactions with the ribosome’s GAC may additionally stabilize the active conformation of the catalytic centre in the G domain and trigger GTP hydrolysis in the presence of the ribosome.

Upon EF-G binding and GTP hydrolysis, the ribosomal 30S subunit rotates counter-clockwise with respect to the 50S subunit in a ratcheting motion [95, 110-111], which is accompanied by a concerted movement of the A and P site tRNAs, initially with respect to the 50S, but not 30S, subunit, also known as the “hybrid state” [112-115]. The acceptor end of the A site tRNA, which now carries the polypeptide chain, rapidly reorients and moves into the P site of the 50S subunit, while its anticodon stem remains bound to the 30S A site. Similarly, the now deacylated P site tRNA also reorients and places its acceptor arm into the 50S E site on the 50S subunit, while its anticodon loop remains attached to the 30S P site. Thus, the previously A/A peptidyl-tRNA is now in the A/P site, while the previously P/P deacylated tRNA has moved in the P/E site. At this point, r-protein L1 is believed to reach almost 20 Å into the P/E site and contact the elbow of the deacylated tRNA, which is the first step in the L1 directed exit mechanism for E site tRNAs.

In order to complete translocation, Pi release from EF-G is needed to trigger further structural rearrangements of both EF-G and the ribosome [97, 116-

122]. Upon ribosomal ratcheting and tRNA translocation, EF-G extends and rotates domains III, IV and V onto domains I and II, such as to place the tip of domain IV in the ribosomal A site (also see Fig. 1.5.), which probably helps push the A/P and P/E tRNAs to the P/P and E/E sites respectively, while also preventing their back-translocation. As EF-G extends, it “wedges” the ribosomal subunits, so that the inter-subunit contacts (bridges) between the 30S head and the 50S protuberance, which involve both rRNA and r-proteins S13, S19 and L5, rearrange to allow the rotation of the head and spur of the 30S with respect to the 50S ribosomal subunit. This rotation is conducted by helix 44 of the 16S rRNA, which moves towards the P site, while all along maintaining contact with EF-G. Also, domain III of EF-G is in contact with S12, which may help the rotation of helix 44. Consequently, the two tRNAs translocate onto the 30S subunit, dragging the mRNA along by one codon. Next, the 30S subunit undergoes a second, reversed, set of movements which culminate with the 30S ratcheting back to its original position, as the L1 stalk pulls the deacylated tRNA into the E/E site [95]. Similar to peptide bond formation and translocation, inter-subunit ratcheting has been shown to also be an intrinsic property of the ribosome, which is accelerated by EF-G, probably by lowering a kinetic barrier for ratcheting, since ribosomal subunit rotation has also been shown to take place in the absence of EF-G and charged tRNAs [123].

Following GTP hydrolysis and 30S translocation, switch I of EF-G flips outward from the ribosomal cavity, which favors GDP release and EF-G dissociation from the ribosome [99]. In solution EF-G exchanges spontaneously GDP for GTP, as it has slightly higher affinity for GTP than GDP (by approximately two fold [101]), plus in healthy cells, GTP is predominant over GDP.

Following complete translocation, the E site now contains the deacylated tRNA, the P site holds the peptidyl-tRNA, the A site is empty, and the ribosome is unratcheted. The next step is EF-Tu binding to the F site to deliver the next aa-tRNA corresponding to the A site mRNA codon in the empty A site. The elongation cycle is repeated until a stop codon reaches the A site.

I.2.3. Termination

Bacterial translational termination (Fig. I.8., reviewed in [124]) is carried out by RFs, with some additional help from EFs and IFs. Class I RFs (RF-1 and RF-2) catalyze peptide release, while class II RFs (RF3) remove the class I RFs from the ribosome. In eukaryotes, there is only one class I RF (eRF-1 in eukaryotes and mRF-1 in mitochondria) which recognizes all three stop codons.

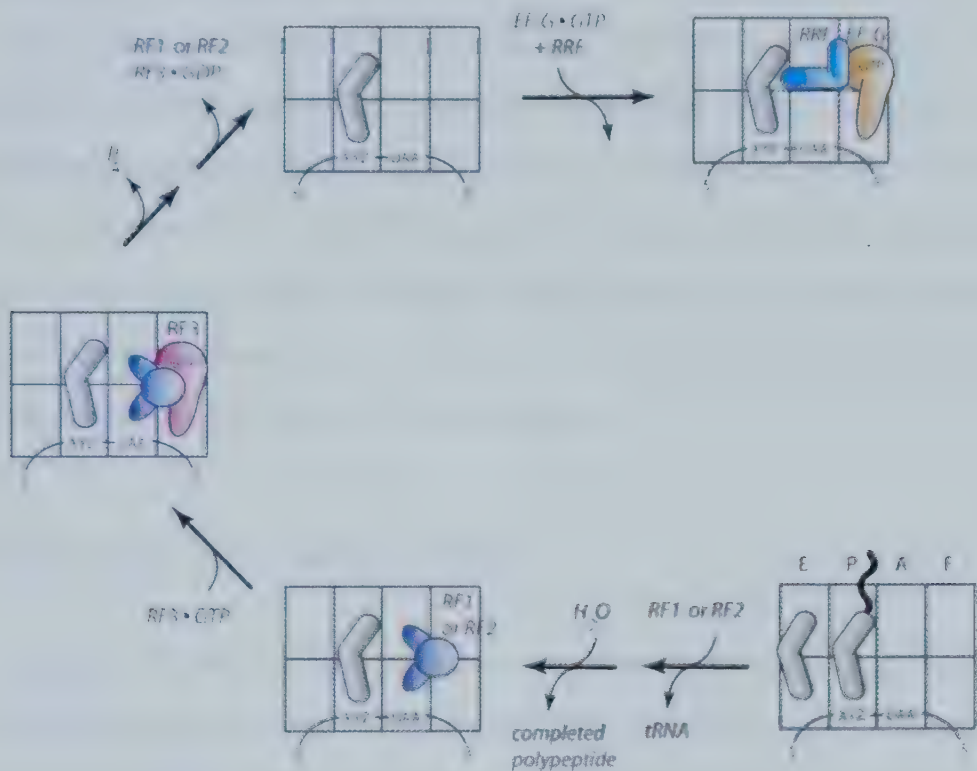


Fig. I.8. Bacterial translation termination

The schematic representation of the ribosome, tRNAs, mRNA and EF-G is the same as in Fig. I.3. The RFs and RRF are as marked.

In bacteria, the three stop codons are specifically recognized by RF-1 (UAA and UAG) and RF-2 (UAA and UGA). **RF-1** and **RF-2** have a four domain structure, out of which domain II contains a putative tripeptide “anticodon” motif, P(A/V)T in RF1 and SPF in RF2, which contacts the stop codon, especially at the second and third base positions. Domain III contains a conserved GGQ motif which reaches into the PTC and makes contact with U2585 and A2602 of the 23S rRNA, while also facing A76 of the P site peptidyl-tRNA. The GGQ mediates the hydrolysis of the ester bond between the tRNA and the peptidyl chain. Domain I

sticks out of the ribosome and interacts with the L11 region. **RF3** is a GTPase, structurally similar to EF-Tu, which binds to the above RF-1/RF-2-ribosome complex in the GDP form. Polypeptide chain release by class I RFs stimulates RF-3 to exchange GDP for GTP and adopt a more stable conformation on the ribosome, which also involves contacts with L11, believed to trigger GTP hydrolysis by RF-3. Following GTP hydrolysis, RF-3·GDP and RF-1/RF-2 dissociate from the ribosome.

Then, the ribosome recycling factor (RRF in bacteria and eRRF in eukaryotes) and EF-G (or eEF-2, respectively) recycle the ribosome for the next round of translation in a process that involves splitting apart of the two subunits with the assistance of IF-3 (or eIF-1 and eIF-1A, respectively). Subsequently, the tRNA and mRNA are released, while IF-3 associates with the 30S to maintain the ribosomal subunits dissociated, which in turn promotes the binding of the other IFs, of the initiator tRNA and of the new mRNA.

I.3. INHIBITORS OF TRANSLATION

Antibiosis (“against life”) is a natural phenomenon first described in 1877 in bacteria by Louis Pasteur and Robert Koch, by which natural substances or compounds are able to kill, or inhibit the growth of, bacteria. These biological products were later renamed “antibiotics” in 1942. Today, synthetic compounds have been additionally developed to complement and improve the antibiosis effects of natural substances.

Although generically, “antibiotic” is the term used to delineate compounds used in medical practice to treat bacterial infections in humans or animals, there are antibiotics which have been found to possess indiscriminating toxicity that would be harmful for both the host and the bacteria, thus they are not used in clinical settings. Nevertheless, in laboratory controlled environments, these toxic compounds have proven to be invaluable research tools for understanding the underlying mechanistic details of translation, while at the same time providing key information for intelligent antibacterial drug design.

The translation machinery in general, and the ribosome in particular, have long been known to be targets for many classes of bacterial growth inhibitors (reviewed in [125]), which act at almost every step of translation. Here I review only the translational inhibitors most relevant to this thesis' research.

1.3.1. Selected inhibitors of translation

Kirromycins bind between domains I and III of EF-Tu and trap the ternary complex on the ribosome by preventing the conformational changes of the EF-Tu switch regions required for GTP hydrolysis.

Tetracyclines bind on the head of the 30S subunit, where they interact with helices 31 and 34, thus preventing the stable binding of the EF-Tu ternary complex to the ribosome by directly overlapping with the anticodon stem-loop of the tRNA.

Aminoglycosides are well known to stimulate misreading at the 30S decoding site, while also being strong translocation inhibitors and back-translocation promoters. Miscoding effects similar to those caused by aminoglycosides have been observed by mutating r-protein S4, without affecting cell viability, which suggests that aminoglycoside effects on decoding are bacteriostatic rather than bactericidal. Most aminoglycosides bind within an internal loop in helix 44 of the 30S subunit, which comprises the decoding site. In doing so, they favor near-cognate tRNAs binding to the A site by inducing and stabilizing the flipped-out conformation of A1492 and A1493 in the decoding centre. Hygromycin B is an atypical aminoglycoside, which binds to helix 44 of the 30S subunit, and although exerting only a modest effect on translational fidelity, directly contacts the mRNA in both P and A sites, thus confining the mRNA on the ribosome, and in turn, preventing translocation.

Macrolides bind within the exit tunnel of the large ribosomal subunit and prevent elongation of the nascent polypeptide chain by blocking its passage through the tunnel. In most cases, this leads to a drop-off of the peptidyl-tRNA from the ribosome.

Thiostrepton inserts into a cleft formed by the NTD of r-protein L11 and helices 43 and 44 of the 23S rRNA, which overlaps with the binding site of translation factors, like IF-2, EF-Tu and EF-G. With respect to EF-G, thiostrepton prevents the movement of L11-NTD which opens the cleft for the insertion of domain V of EF-G during accommodation. Thus thiostrepton may not inhibit initial binding of EF-G, nor GTP hydrolysis, but instead prevents Pi release and translocation by trapping the factor on the ribosome [115, 123-124].

Ribotoxins are natural proteins produced by bacteria, fungi and plants, to damage the ribosomes of other organisms, either prokaryotic or eukaryotic. The most widely known factors of this family, α -sarcin (bacteria) and ricin (eukaryotes) target helix 95 of the 23S rRNA, hence its name of the sarcin-ricin loop (SRL). The SRL is one of the most highly conserved regions of the ribosome and comprises a continuous irregular helix closed by a GAGA tetraloop, of which the two bases A2660 and G2661 are exposed at the tip. α -sarcin cleaves the SRL on the 3' side of G2661, while ricin hydrolyzes the N-glycosidic linkage between the ribose and sugar at the nucleotide corresponding to *E. coli* A2660 in eukaryotic ribosomes, thus removing the purine base. Cleavage of the SRL by ribotoxins completely abolishes GTP hydrolysis in EF-Tu and EF-G, and in turn, protein synthesis.

Puromycin (Fig. I.9., A.) binds to the PTC, overlapping the aminoacyl moiety of the A site aa-tRNA, which protects the peptidyl-tRNA from hydrolysis, and in turn blocks the ribosomes on the mRNA. Since puromycin inhibits cellular growth in all three kingdoms, it is primarily used as a research tool for studying peptide bond formation.

Viomycin (Fig. I.9., B.) can trap the ribosome in the ratcheted state during translocation [127]. It can also induce back-translocation [128] and translational misreading [129], analogous to aminoglycosides. The viomycin binding site is also likely to overlap with that of aminoglycosides, since these drugs compete with each other for ribosome binding.

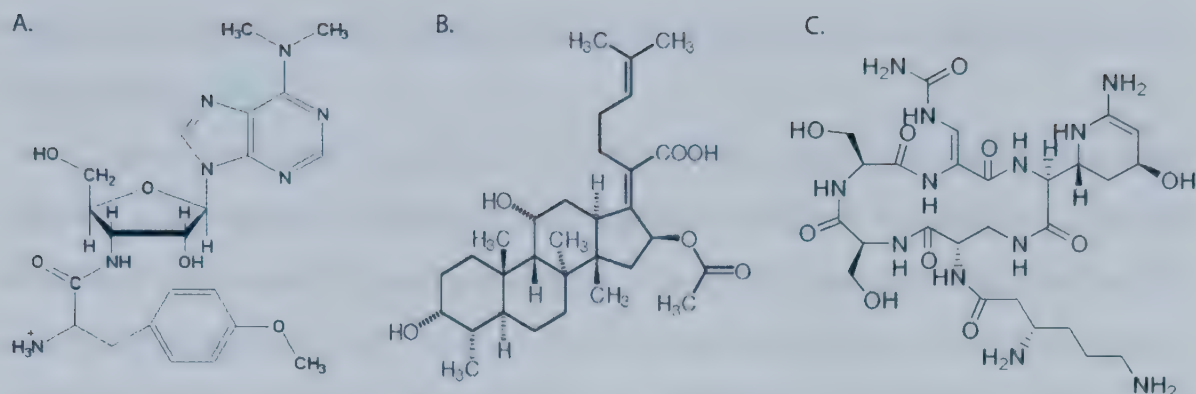


Fig. I.9. Chemical structures of selected translational inhibitors.

A. puromycin.

B. Fusidic acid.

C. Viomycin.

Fusidic acid (Fig. I.9., C.) is a steroidal antibiotic produced by the fungus *Fusidium concineum* that targets only bacterial ribosomal machineries, hence its introduction into clinical use in 1962 [130-131], mainly for the treatment of *Staphylococcus aureus* (*S. aureus*) infections [132]. Fusidic acid binds at the interface between domains I and III of post-translocationally ribosome bound EF-G [12], and prevents progression of protein synthesis by blocking EF-G release from the ribosome [99, 133-134].

I.3.2. Antibiotic resistance and research tools

Partially due to antibiotic misuse, but mostly due to evolutionary selection processes, bacteria have developed efficient antibiotic resistance mechanisms, with the ones most commonly employed being: (1) drug inactivation or modification, (2) alteration of the target site, (3) alteration of the metabolic pathways involved in the bactericidal effects, and (4) decreasing drug accumulation, by reducing influx and/or increasing efflux across the cell surface.

Although bacteria often employ more than one of these mechanisms against a given antibiotic, target site alteration is one of the most often employed antibiotic resistance mechanisms and comprises spontaneous mutations or modifications (i.e. methylation) of the rRNA (aminoglycosides, macrolides,

viomycin, etc.) or of amino acid residues of EF-Tu (e.g. kirromycins) or EF-G (e.g. fusidic acid).

Here are some examples of antibiotic resistance mechanisms involving slightly more elaborate pathways. For example, a variant of metabolic pathway alteration mechanism is the tetracycline resistance mechanism, which involves the use of ribosome protection proteins (RPPs), such as Tet(O) or Tet(M) (reviewed in [135]). RPPs are GTPases that bind to the ribosome analogously to EF-G and chase the antibiotic off the ribosome. Also, macrolides have been shown to correlate their translational stalling ability with the sequence of the leader peptide in the tunnel, with the translation of some amino acid sequences even leading to antibiotic removal from the ribosome [136].

Although the emergence of resistant bacterial strains poses serious health dangers, in a controlled laboratory environment, antibiotic resistance constitutes an important research tool. The antibiotics used in genetic engineering are generally "older" antibiotics which have largely fallen out of use in medical clinical practice, such as ampicillin, kanamycin, tetracycline and chloramphenicol. As an example, genetic engineering methods can introduce antibiotic resistance plasmids into a bacterial population of interest, i.e. carrying a gene of interest. Upon subsequent exposure of the bacterial population to the respective antibiotic, only the sub-population expressing the antibiotic resistance plasmid and the gene being manipulated will survive in the antibiotic-containing media.

1.4. THESIS OVERVIEW

As GTPases, EF-G and EF-Tu share two conserved mobile structural elements within the G domain, called switch I and switch II, which are believed to act by molecular spring mechanisms to unlock the transition between the factors' functional states on the ribosome [137]. During their catalytic cycles, EF-G and EF-Tu undergo significant and different conformational changes, which are

believed to be triggered/facilitated by rearrangements of the switch regions upon GTP hydrolysis [30, 95, 106-107, 109].

We investigated the role of these two switch regions in EF-G and found that switch II and its contacts with the interface between domains I and III are essential for EF-G cycle progression from ribosome bound to activation of GTP hydrolysis, to translocation. We also described the positional change of switch I upon GTP hydrolysis and 30S translocation, and proposed that such conformational rearrangement of the switch I region may be involved in nucleotide release and EF-G recycling.

Our study of the switch regions of EF-G also uncovers mechanistic details of fusidic acid resistance, as the interface between switch I, switch II and domain III has been shown to host the fusidic acid binding site [12], as well as a plethora of spontaneously arisen fusidic acid resistant mutations [106-107, 109].

We also investigated EF-P's overall role in translation as well as its functional interactions with EF-G. We found that bacterial EF-P has no influence on EF-G's function, nor does EF-G exert a detectable effect on EF-P's known ability [138] to stimulate peptide bond formation. Our results suggest that EF-P is not an initiation factor *per se*, nor is it involved in elongation, but rather functions at an intermediate step between the two steps of translation. This work can be understood from a functional perspective in the light of a complementary structure of EF-P on the ribosome [28].

Our specific studies on EF-G and EF-P have enriched our understanding of complex interrelationships between the ribosome and its core translation factors during protein synthesis. Furthermore, the projects described in this thesis also have implications for antibiotic sensitivity and resistance mechanisms.

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**II. SWITCH II AND THE INTERFACE BETWEEN DOMAINS I AND
III OF EF-G IS ESSENTIAL FOR THE PROGRESSION FROM GTP
HYDROLYSIS TO TRANSLOCATION ON THE RIBOSOME**

II.1. INTRODUCTION

EF-G and EF-Tu share certain structural and functional similarities. As GTPases, both factors share two conserved structural elements, termed “switches”, in their G domains [1]. To date, EF-Tu is better characterized than EF-G with regard to its intrinsic structural rearrangements (reviewed in [2-3]), while EF-G, although extensively studied in terms of function (reviewed in [4-5]), and despite recent progress in solving its ribosome bound post-translocational structure [6], has yet to be characterized with regard to its dynamic internal structural rearrangements in the pre-translocational state.

An increasing body of evidence indicates that EF-G changes conformation cooperatively with the ribosome, rearrangements which most likely “unlock” the progression of its catalytic cycle. To begin with, the crystal structures of EF-G in the absence of the ribosome [7-8] do not superimpose the GTP structure of EF-G on the ribosome [9], as ribosome-bound EF-G appears to have a different orientation of domain III relative to domains I and II, most likely due to altered interactions between the switch regions in domain I and domain III residues. A recent 3.6 Å resolution post-translocational crystal structure of EF-G on the ribosome [6] supports previous cryo-EM studies which hypothesized that EF-G, in cooperation with the ribosome, would undergo essential rearrangements of domains III, IV and V with respect to domains I and II [10], which converge to extend the EF-G molecule and position of the tip of domain IV into the 30S A site. Thus EF-G would function as a “wedge” to advance the tRNAs and mRNA towards the ribosomal E and P sites, while probably secondarily preventing frame shifting and back-translocation [11].

Additional structural information comes from the interaction of EF-G with the antibiotic fusidic acid. Initial studies indicated that the antibiotic is able to trap EF-G·GDP on the ribosome immediately after the first round of GTP hydrolysis [12]. However recent smFRET experiments suggest that EF-G is able to undergo several rounds of GTP hydrolysis before being irreversibly trapped on the ribosome in complex with fusidic acid [13]. Fusidic acid only binds to a post-

translocational EF-G-ribosome complex [14], and not to EF-G in solution [12], suggesting that the structure of EF-G changes during ribosome binding and translocation, making it accessible to fusidic acid. The recent crystal structure of EF-G trapped by fusidic acid on the ribosome [6], confirms the position of the antibiotic at the interface between domains I and III [15], where it appears to be in close contact with domain III residues as well as with switch II of EF-G [6]. The tip of switch II harbors the strongest of many spontaneously arisen fusidic acid resistant mutants, initially identified and characterized in *S. aureus* [16] and *Salmonella typhimurium* (*S. typhimurium*) [17]. Interestingly, crystallography studies of fusidic acid dependent mutants in the absence of the ribosome [15, 18] show that the position of the tip of switch II in a fusidic acid sensitive mutant adopts a diametrically opposite position compared with fusidic acid resistant mutants, rotating away from domain III and packing against a hydrophobic pocket in domain V. This orientation appears to provide less stabilization of domain III (and especially of helix B_{III}), suggesting that the interactions between switch II and domain III may affect the overall conformation of EF-G as well as its sensitivity/resistance to fusidic acid.

Similar to fusidic acid, the antibiotic sordarin inhibits EF-2-mediated translocation by acting at a step which precedes GTP hydrolysis [19]. The crystal structure of EF-2 in solution resembles that of EF-G·GDP, however upon sordarin binding, probably to domain III of EF-2 [20], domains I and II remain rigid, while domains III, IV and V rotate as separate blocks [21]. Similar movements of EF-2 have been reported by cryo-EM studies [22-23].

In order to investigate the role of the interface between domains I and III of EF-G, we have mutated seven residues situated in domains I and III to alanines and probed their activity by comparison with the wild type protein during key steps of the EF-G cycle. We found that the inter-domain region formed by the tip of switch II and residues in domain III may act as a conformational “joint”, which functions by a “ball and socket” rotational mechanism, thus modulating essential conformational changes of EF-G necessary for the progression of EF-G’s translational cycle.

II.2. RESULTS

II.2.1. Examining the interface between domains I and III of EF-G

In the absence of the ribosome, single point fusidic acid resistant/sensitive mutations in domain I have been associated with intrinsic rearrangements of the residue situated at the tip of switch II, F95 (*E. coli* numbering is used throughout this chapter), with respect to helix B_{III} in domain III of EF-G. These intrinsic rearrangements eventually translate into an overall more compact shape of the EF-G molecule [15, 18]. F95 also harbors the strongest spontaneously arisen fusidic acid resistant mutant identified to date, (F95L) [15] and, as shown in the fusidic acid complex of EF-G on the ribosome [6], the phenyl ring of F95 directly contacts fusidic acid. Domain III residues (and helix B_{III} residues in particular) situated at the interface with domain I, also harbor spontaneous fusidic acid resistant mutants [15].

When comparing the GTP and GDP structures of ribosome bound wild type EF-G [6, 9], F95 has been shown to maintain a constant orientation towards domain III, suggesting it may function as a pivot for the rotation of domains III, IV and V as the EF-G cycle progresses from GTP hydrolysis into translocation; helix B_{III} residues in domain III may act as a hydrophobic stabilization surface for switch II at the inter-domain interface. Interestingly, domain III deletion studies reveal that domain III is essential for ribosome activated GTP hydrolysis [24], while structural studies reveal it to make intimate contacts with both subunits of the ribosome, thus possibly “sensing” whether the ribosome is in a ratcheted or non-ratcheted state [6], e.g. “ready” for GTP hydrolysis by EF-G.

In order to examine the interactions between switch II and domain III, we examined residue F95 in switch II (domain I) and six other conserved residues in domain III, which remain oriented towards F95 in the GTP and GDP structures of EF-G on the ribosome [6, 9]: D442, M461, L464, H465, I468 and R472 (Fig. II.1., A.). A theoretical model using Pymol software, surface mode, shows that the interface between domains I and III of EF-G is lined by a network of interactions involving these seven residues (Fig. II.1., B.).

Using the Pymol software, residue mutagenesis mode, we changed all seven residues to alanines (Fig. II.1., C.), and re-examined the inter-domain interactions. Interestingly, we found that the surface interactions between switch II and domain III involving residues F95, D442, R472 and I468 become interrupted (Fig. II.1., D.).

Overall, this modeling prompted us to create all seven individual alanine mutants in *E. coli* EF-G.

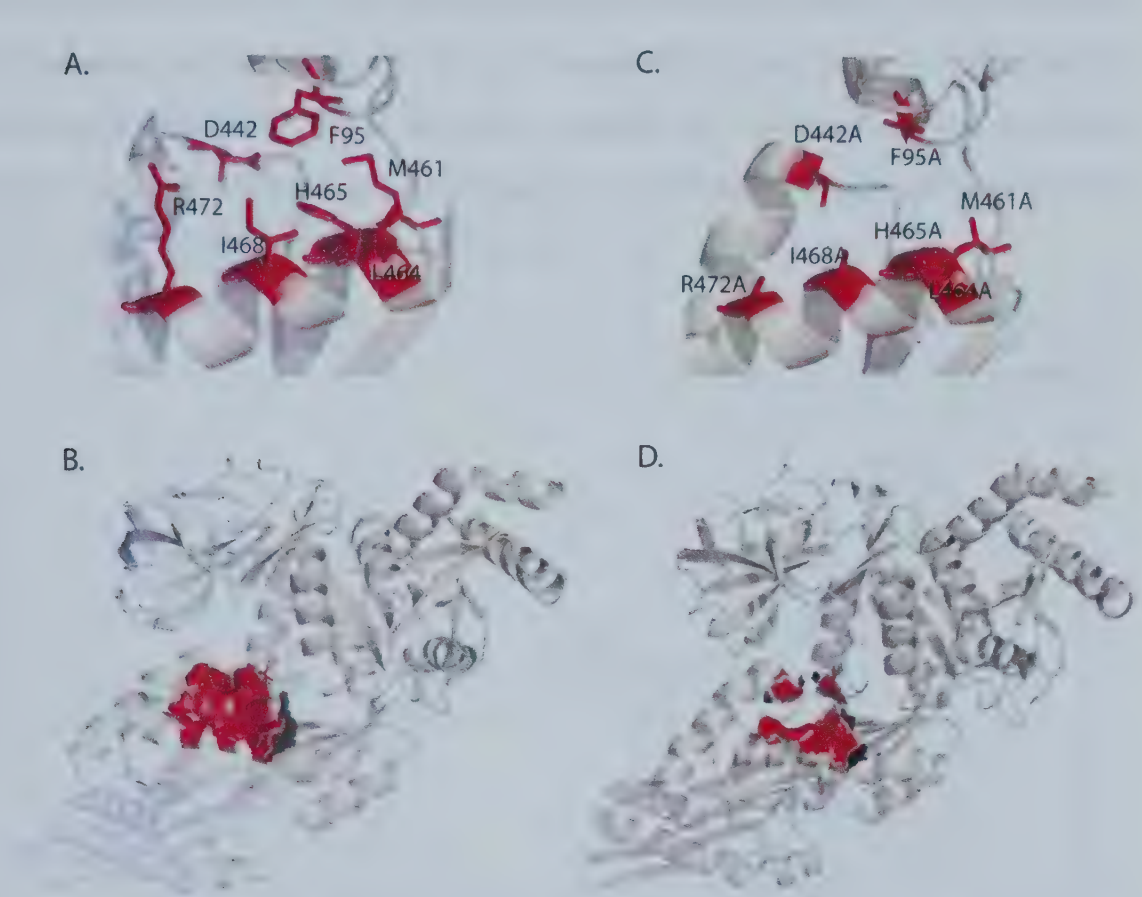


Fig. II.1. Examining the interface between domains I and III (PDB file 2OM7)

- A. F95 is surrounded by residues in domain III.
- B. Pymol surface view mode was applied to the residues depicted in panel A.
- C. Pymol software was used to mutate all residues from panel A. to alanines.
- D. Pymol surface view mode was applied to the residues depicted in panel C.

II.2.2. Some mutants exhibit unusual GTP binding and hydrolysis behavior

In order to probe our proteins in terms of their nucleotide binding affinity, we used two fluorescently labeled nucleotides, mant-GTP and mant-GDP. Since increased hydrophobicity of the environment surrounding the mant group translates in fluorescence increases, we measured the fluorescence resonance

energy transfer (FRET) between tryptophan residues situated close to the GTPase center of EF-G and the mant group of the two nucleotides, as described [25]. EF-G wild type exhibited k_{ds} of $7 \pm 2 \mu\text{M}$ for mant-GTP and $11 \pm 4 \mu\text{M}$ for mant-GDP which is in accord with previously determined values [25] (Table II.2).

Interestingly, in the presence of mant-GTP (and unlike in the case of mant-GDP), all mutants except for H465A (and L464A to a lesser extent) showed non-linear increases in fluorescence upon mant-GTP addition. The rate of the exponential increase tends to plateau was indirectly proportional with the mant-GTP concentration (up to 10-20 μM mant-GTP), while above 20 μM the fluorescence increase. Such kinetic behavior is consistent with enzymatic saturation binding effects. A representative comparative experiment of EF-G wild type and EF-G (F95A) is shown in Fig. II.2.

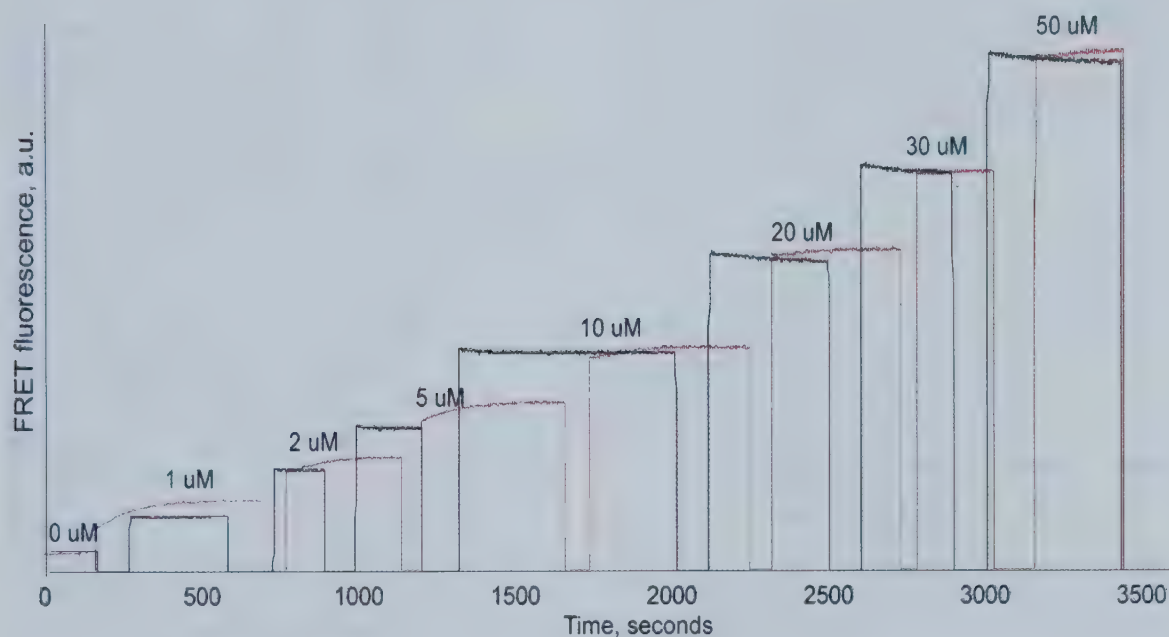


Fig. II.2. Comparing wild type (black) and F95A (red) fluorescence behavior upon titration of mant-GTP

Increasing concentrations of mant-GTP (as indicated) were added to approximately 80 μl reactions containing 1 μM EF-G, and gently mixed by pipeting. The fluorescence recordings are shown above.

We decided to further investigate the molecular basis for these unexpected fluorescence changes. We started off with only mant-GTP in the reaction and, in a step-wise manner, added EF-G (wild type or F95A) and ribosomes to the reaction (Fig. II.3., A. and C.). After each addition, we removed aliquot samples from the

cuvette and separated the mant nucleotides by thin layer chromatography (TLC) (Fig. II.3., B. and D.).

As found previously [26], wild type EF-G showed no detectable change in fluorescence upon mant-GTP addition, while in the presence of ribosomes, mant-GTP hydrolysis was accompanied by a decrease in fluorescence intensity (Fig. II.3., A. and B.). However, unlike wild type EF-G, the F95A mutant showed a fluorescence increase in the presence of mant-GTP (Fig. II.3., C.). TLC analysis of the sample taken from this reaction revealed significant mant-GTP hydrolysis in the presence of F95A and in the absence of ribosomes (Fig. 3, D., sample S3).

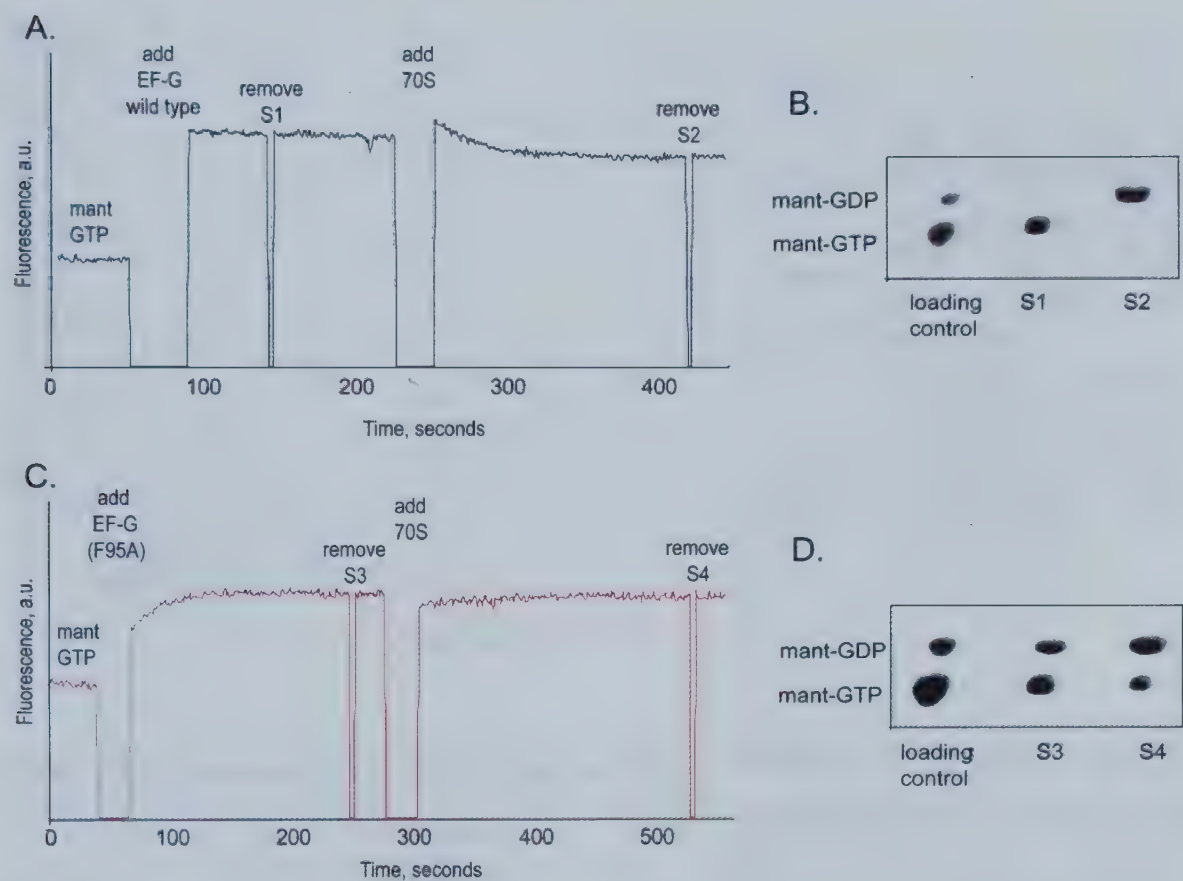


Fig. II.3. EF-G wild type (black) and F95A (red) cause different fluorescence changes in the presence of mant-GTP and ribosomes

A., C. The initial level of fluorescence corresponds to the presence of 120 μ M mant-GTP in the same 80 μ l cuvette as the one used in Fig. II.2.. 25 μ M EF-G were added to the labeled nucleotide, and samples S1 (for wild type) and S3 (for F95A) were extracted. Then 1.4 μ M 70S were added to the reaction and samples S2 (for wild type) and S4 (for F95A) were extracted.

B., D. S1, S2, S3 and S4 were separated on silica gel 60 (F-254) TLC together with loading controls of 500 pmols each of mant-GDP and mant-GTP.

F95A continued to behave differently from wild type upon addition of ribosomes, when the fluorescence levels remained constant, despite continued hydrolysis of mant-GTP (Fig. II.3, D., sample S4).

Since D442A, M461A, I468A and R472A had similar fluorescence behavior as F95A in the presence of mant-GTP, we verified whether they also exhibited un-inhibited intrinsic GTP hydrolysis activity. We probed these proteins under multiple turnover GTP hydrolysis conditions and found that all mutants except for L464A and H465A exhibited increased rates of GTP hydrolysis in solution compared with wild type EF-G (Fig. II.4.).

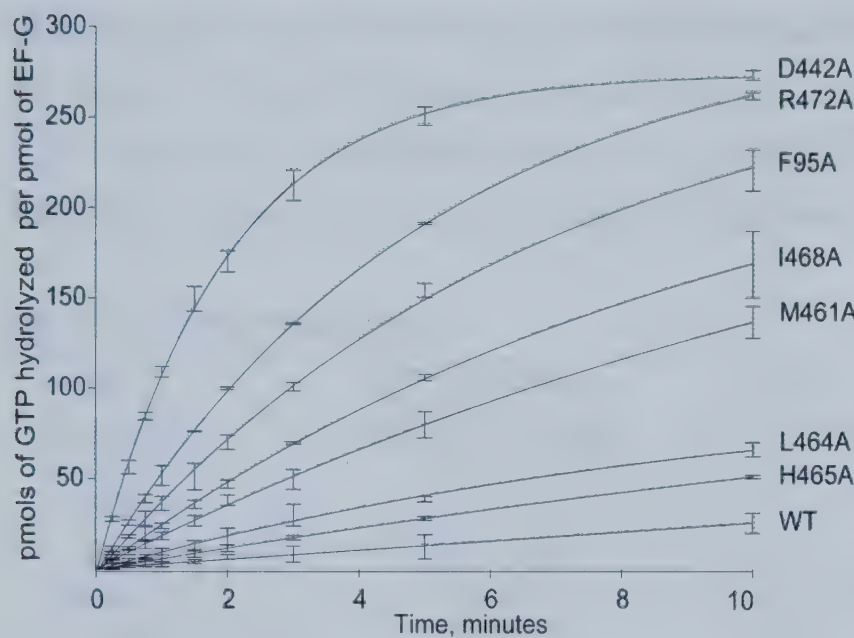


Fig. II.4. Some of the alanine mutants are able to activate GTP hydrolysis in the absence of ribosomes
Multiple turnover conditions: 0.4 μM EF-G and 100 μM GTP (with trace [γ^{32} P]-GTP).

Table II.1. Intrinsic GTP hydrolysis activity

EF-G mutant	Intrinsic GTP hydrolysis rate (10^{-4}s^{-1})
WT	4.3 ± 0.5
F95A	45.5 ± 1.3
D442A	130.3 ± 10
M461A	23.3 ± 1.3
L464A	12.5 ± 1.5
H465A	8.9 ± 0.1
I468A	32.9 ± 3.2
R472A	69.1 ± 1.3

Notably, the mutants forming the theoretical gap observed in Fig. I.1., D. (F95A, D442A, R472A and I468A), exhibited the highest rates of intrinsic GTP hydrolysis (10, 30, 16 and 8 fold higher than the wild type EF-G protein, respectively; see Table II.1.).

Since the fluorescence increase observed for F95A, D442A, M461A, I468A and R472A upon mant-GTP addition correlates with un-inhibited intrinsic GTP hydrolysis activity, one could conclude that such fluorescence changes reflect a combination of mant-GTP binding and hydrolysis. In order to assess the combined kinetics of these reactions, we used a rapid mixing apparatus to measure the k_{app} (a combination of mant-GTP binding and hydrolysis) for F95A, D442A, M461A, L464A, I468A and R472A. L464A exhibited a lower slope of fluorescence increase upon mant-GTP addition than the rest of the intrinsically active alanine mutants.

The k_d values for mant-GTP binding by wild type and H465A, as well as all k_d values for mant-GDP binding were calculated based on the stable levels of fluorescence obtained upon mant nucleotide titration, as described by [25]. The k_{app} values for the other alanine mutants were calculated based on the rates of fluorescence increase observed while rapidly mixing mant-GTP and EF-G.

Table II.2. Nucleotide binding

EF-G mutant	k_d or k_{app} (μ M) for mant-GTP binding	k_d (μ M) for mant-GDP binding
WT	6.9 ± 2.2	11.4 ± 3.6
F95A*	4.1 ± 0.7	10.9 ± 5.1
D442A*	12.6 ± 0.4	19.9 ± 4.1
M461A*	2.7 ± 0.8	11.8 ± 3.9
L464A*	4.9 ± 1.9	10.3 ± 7.4
H465A	39.3 ± 4.2	34.9 ± 7.7
I468A*	13.6 ± 1.5	13.6 ± 4.2
R472A*	7.8 ± 0.7	13.4 ± 4.4

* The determined values reflect k_{app} rather than k_d as these proteins intrinsically hydrolyze mant-GTP up to 30 fold faster than wild type EF-G. Thus the rate we determined reflects both GTP hydrolysis and nucleotide binding.

By examining the calculated k_d and/or k_{app} (Table II.2.), we estimate that the alanine mutants bind both mant-GTP and mant-GDP similar with wild type. However, the mutants that exhibited increased GTP hydrolysis ability in solution appear to also have different behavior than wild type in terms of mant-GTP fluorescence, which may translate into a mutagenesis-dependent altered nucleotide environment within the G domain. Moreover, F95A fluorescence does

not decrease in the presence of the ribosome despite obvious hydrolysis of mant-GTP, suggesting either lack of ribosome binding or deficient GTP hydrolysis activation in the presence of the ribosome.

II.2.3. The alanine mutants bind to the ribosome

In order to assess the ribosome binding affinity of our proteins, we incubated EF-G and ribosomes (in a ration of 1:1) in the presence of GDPNP and employed two different techniques to measure EF-G-ribosome interactions.

We first quantified the degree of protection from DMS modification of 23S rRNA nucleotide A2660 for all proteins (Fig. II.5., A.). The corresponding number of pixels in each DMS protection band were normalized with respect to the level of binding observed for the wild type protein. We found that most mutants bind to the ribosome by roughly 90 % of the wild type value, with F95A and D442A being only slightly above 60 % bound (Fig. II.5., B.)

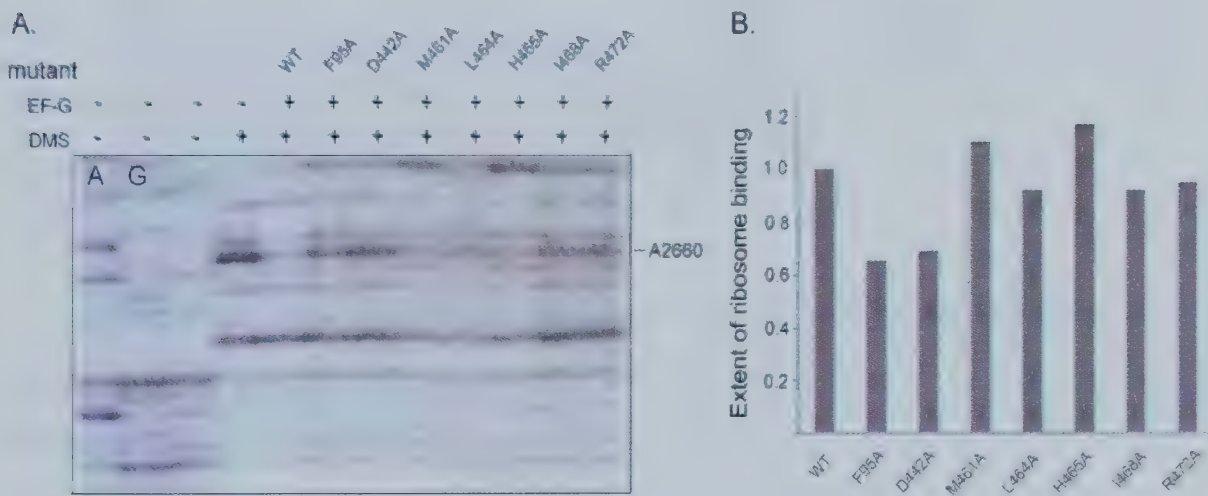


Fig. II.5. Ribosome binding assessment by DMS modification
A. Primer extension techniques detect DMS protection at A2660 in 23S rRNA for all mutants (1 μ M EF-G, 1 μ M ribosomes, 1 mM GDPNP and 33 mM DMS).
B. Quantification of binding based on the degree of protection at A2660 from DMS modification (wild type EF-G was arbitrarily set to equal 1).

Since the mutated region in EF-G (D442 in particular) comes in close contact with the 23S rRNA in the A2260 region of the 50S ribosome [9], and since single residue mutations in this region have been shown to promote structural rearrangements of EF-G [18], it is possible that the previous experiment

may underestimate EF-G binding to the ribosome. Thus we employed an alternative approach to assess ribosome binding.

We treated samples formed under the same conditions as in Fig. II.5. by filtration using a membrane with 100 kDa cut-off limit. Ribosomes and any bound factors would stay on top of the filtration membrane, while molecules smaller than 100 kDa would be lost by filtration. The filtration products were analyzed by SDS-PAGE (Fig. II.6.), confirming that all mutants appear bound to the ribosome, with F95A and L464A exhibiting visually lowest binding.

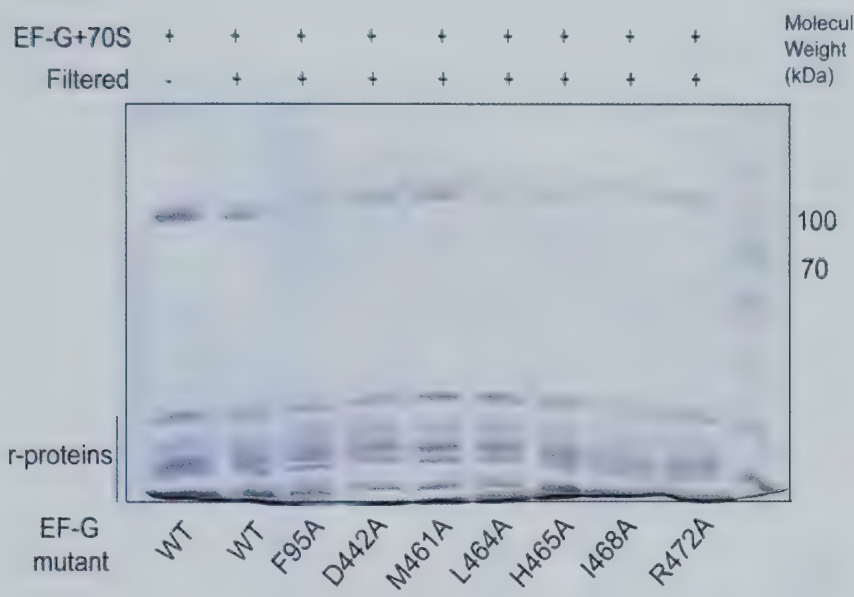


Fig. II.6. Ribosome binding assessment by filtration
10-12 % SDS-PAGE analysis of filtration products of ribosomal complexes formed as in Fig. II.5.

Together, these binding experiments suggest that under stoichiometric conditions, all alanine mutants are at least 50-60 % bound to the ribosome compared with wild type EF-G.

II.2.4. The alanine mutants show impaired activation of GTP hydrolysis in the presence of the ribosome

Since some of the alanine mutants were un-inhibited to actively hydrolyze GTP in the absence of the ribosome, we wondered whether their behavior in the presence of ribosomes exhibits proportionally increased values of GTP hydrolysis. For these purposes, we conducted and quantified multiple turnover GTP hydrolysis reactions in the presence of ribosomes for all proteins.

As shown in Fig. II.7., the ribosome activated GTP hydrolysis ability of all mutants was significantly lower than that of the wild type protein. M461A, L464A and H465A (situated away from the theoretical gap in Fig. II.1., D) exhibited clear activation of GTP hydrolysis in the presence of ribosomes. In contrast, the mutants forming the Fig. II.1., D. gap, F95A, D442A, I468A and R472A, showed poor ability to function cooperatively with the ribosome to activate GTP hydrolysis. In fact, D442A and R472A showed impairment of GTP hydrolysis in presence of ribosomes compared with their respective intrinsic values.

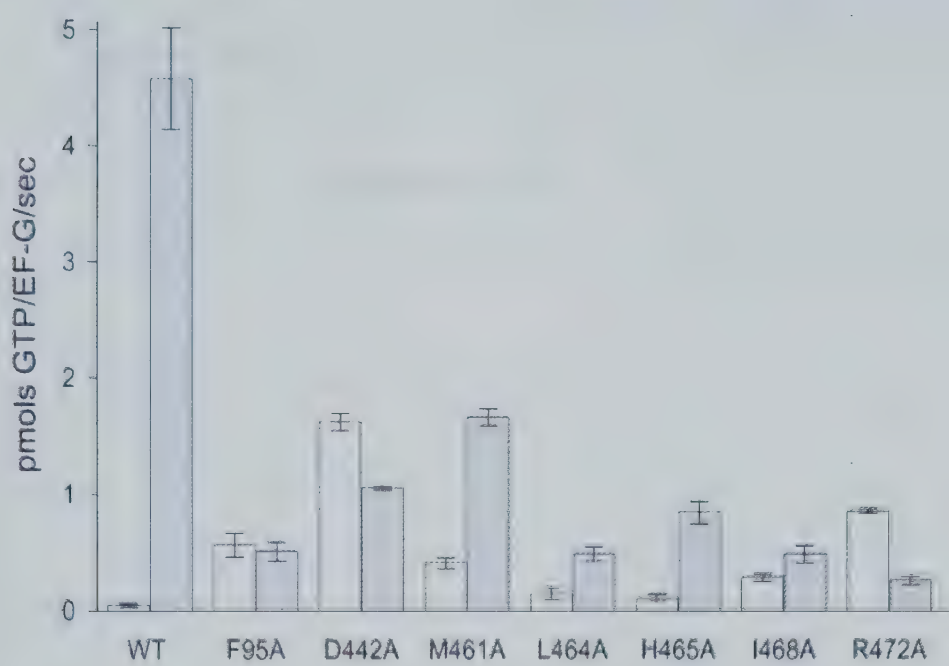


Fig. II.7. Comparing the alanine mutants and the wild type protein in terms of intrinsic and ribosome-activated GTP hydrolysis activity

100 μ M GTP were mixed with 0.4 μ M EF-G in presence (gray) or absence (white) of 2 μ M 70S. Reaction rates were calculated using an average of five time points and final averages and standard deviations were calculated from three independent experiments.

To assess whether the effects we observed for F95A, D442A, I468A and R472A are dependent on ribosome concentration, we determined the ribosomal turnover rates of GTP hydrolysis for all alanine mutants. Consistent with the above results, F95A, D442A, I468A and R472A showed no ribosome dependence of GTP hydrolysis, while M461A, H465A and L464A were impaired in terms of ribosome activation of GTP hydrolysis compared with wild type (Fig. II.8.).

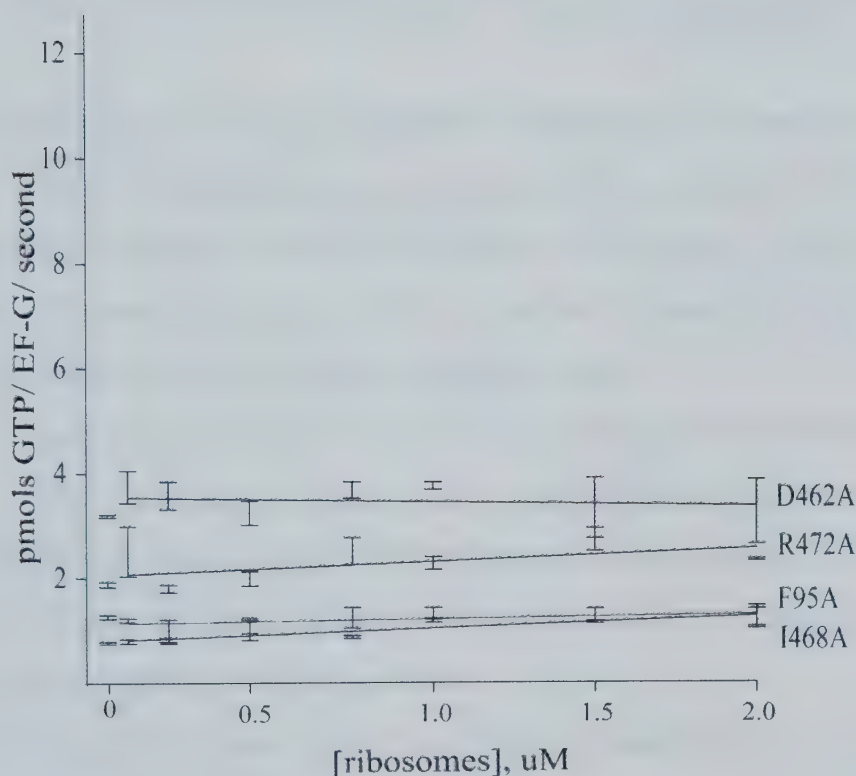
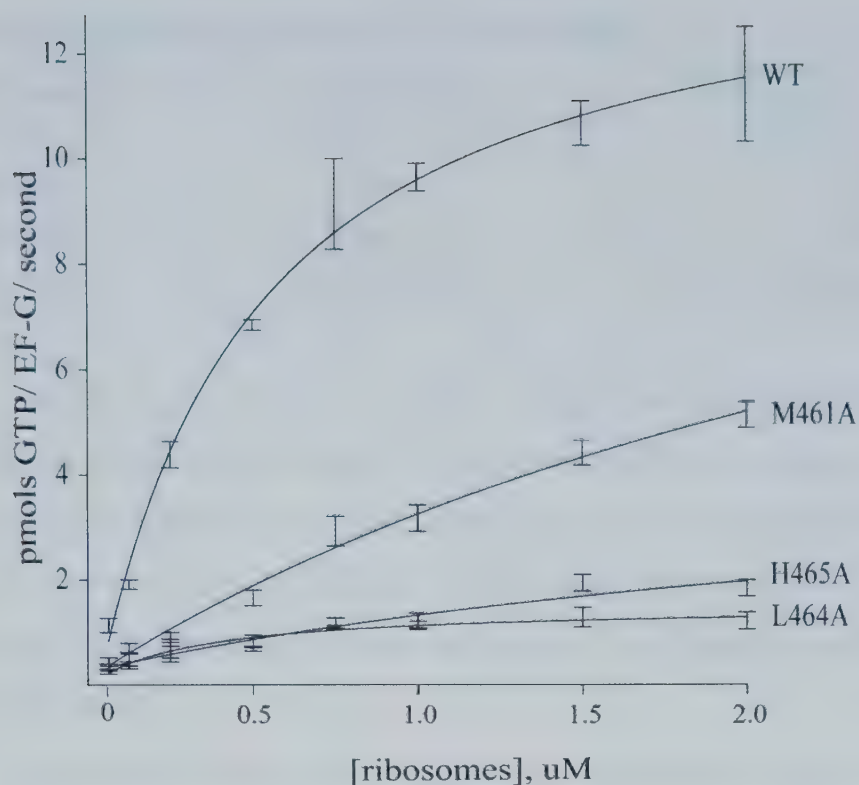


Fig. II.8. Examining ribosome dependence of GTP hydrolysis activation

Multiple turnover GTP hydrolysis conditions: 0.4 μ M EF-G, 100 μ M GTP (with trace $[\gamma^{32}\text{P}]\text{-GTP}$) and various 70S ribosomes concentrations as indicated. Reaction rates were calculated using an average of five time points and final averages and standard deviations were calculated from three independent experiments.

Table II.3. Ribosome-activated GTP hydrolysis

EF-G mutant	K_M (μ M)	k_{cat} (s^{-1})
WT	0.5 ± 0.1	15.7 ± 0.4
M461A	3.9 ± 2	5.1 ± 1.3
L464A	0.4 ± 0.2	1.1 ± 0.8
H465A	2.7 ± 0.6	4.1 ± 0.5

As shown in Table II.3., L464A showed similar ribosome binding ability as wild type, while H465A and M461A had higher K_M values. L464A showed over 10 fold impairment in GTP hydrolysis on the ribosome, while H465A and M461A were only 3-4 fold less active than wild type in the presence of ribosomes. The other alanine mutants showed no ribosome dependence of GTP hydrolysis ability.

In summary, these data suggest that the alanine mutants are impaired in their elongation cycle progression from ribosome binding to GTP hydrolysis.

II.2.5. F95A shows the most significant impairment in translocation

Next, we qualitatively and quantitatively assessed the mutants' ability to translocate 70S-mRNA-tRNAs complexes with respect to the 30S ribosomal subunit. We used toeprinting [27] as a qualitative assay, and pyrene fluorescence-based translocation [26, 28] as a quantitative assay.

Toeprinting was used to measure the extent of translocation for all proteins (Fig. II.9.). Comparative with wild type, M461A and H465A showed no impairment in translocation, D442A, L464A, I468A and R472A translocated to 43, 19, 69 and 65 % respectively. Interestingly, F95A showed only 8 % of the translocation ability of wild type EF-G.

The pyrene-based assay was used to measure the rate of translocation. There are two identifiable components of the decrease in the fluorescence of the pyrene group attached to the 5' end of the mRNA upon 30S translocation: a faster 1st rate (k_{obs1}) and a slower 2nd rate (k_{obs2}) of translocation [26]. As shown in Fig. II.10., wild type EF-G exhibited translocation kinetics as previously reported [26, 28], while the other mutants showed slower translocation rates.

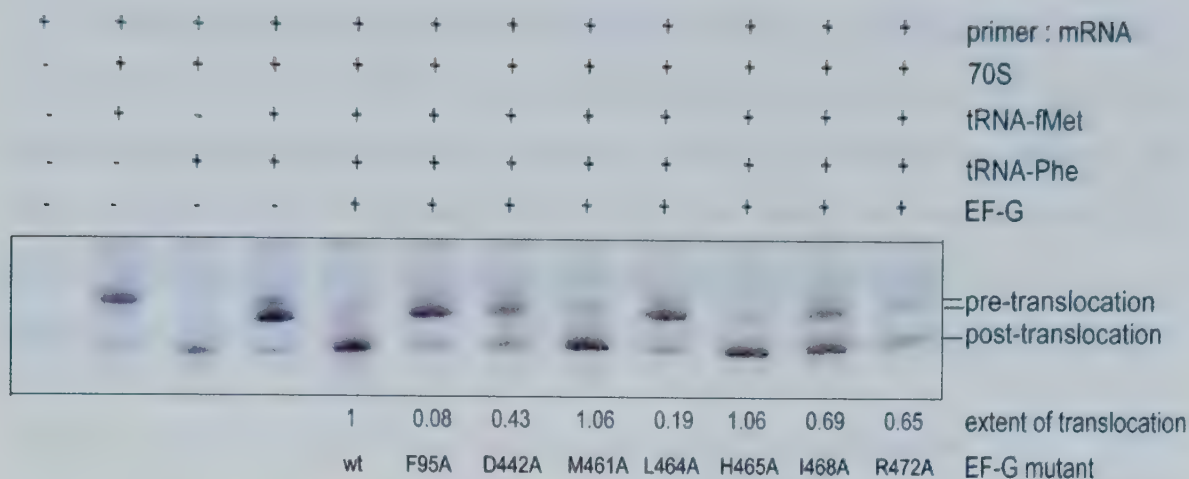


Fig. II.9. Qualitative assessment of single round translocation

Preformed complexes of 1 μ M ribosomes, 1 μ M mRNA, 1.25 μ M tRNA^{fMet} (P site) and 1.25 μ M tRNA^{Phe} (A site) were incubated for 10 minutes with 1.25 μ M EF-G and 2 mM GTP. The extent of translocation was reported with report to the total translocation extent observed for wild type (arbitrarily set at 1).

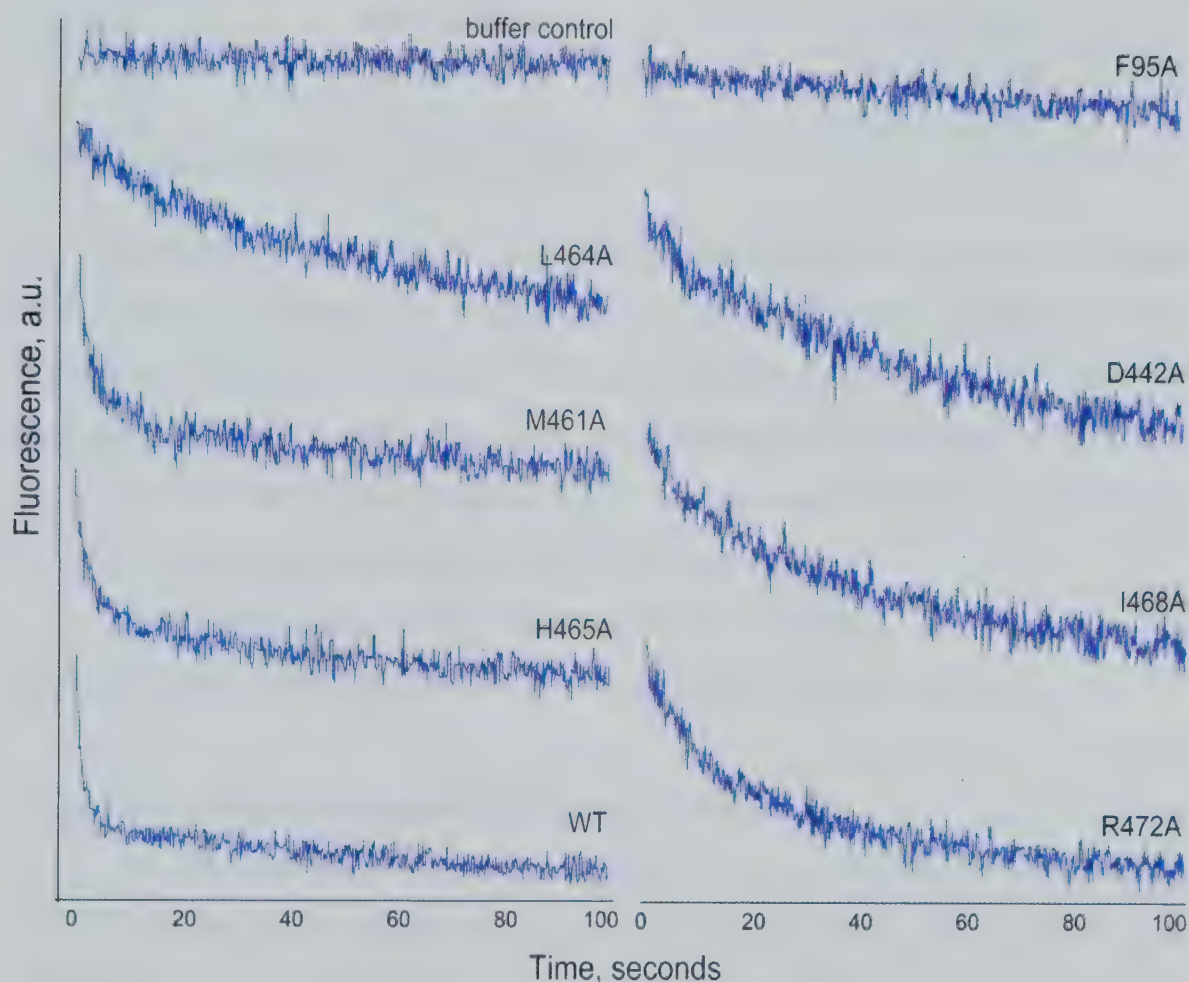


Fig. II.10. Quantitative assessment of single round translocation

Pre-formed complexes of 0.2 μ M pyrene-labeled mRNA oligonucleotide, 0.25 μ M 70S, 0.3 μ M tRNA^{fMet} (P site) and 0.3 μ M tRNA^{Phe} (A site) were rapidly mixed with of 2.5 μ M EF-G and 1 mM GTP.

Both rate components of translocation could be calculated for all mutants except F95A, which exhibited too slow translocation kinetics to be fitted to a double exponential equation. As shown in Table II.4., except for M461A and H465A, which behaved similarly to wild type, the alanine mutants showed up to 40 fold inhibition in k_{obs1} and approximately 20 fold inhibition in the k_{obs2} . By comparison, F95A showed almost 450 fold inhibition of translocation kinetics.

Table II.4. Pyrene-based fluorescent single round translocation

EF-G mutant	1 st exponential (k_{obs1} , s ⁻¹)	2 nd exponential (k_{obs2} , s ⁻¹)
WT	1.806 ± 0.369	0.118 ± 0.023
F95A*	0.004 ± 0.001	-
D442A	0.046 ± 0.002	0.005 ± 0.0005
M461A	0.814 ± 0.137	0.118 ± 0.025
L464A	0.035 ± 0.007	0.004 ± 0.001
H465A	0.892 ± 0.391	0.119 ± 0.057
I468A	0.048 ± 0.003	0.004 ± 0.001
R472A	0.074 ± 0.007	0.005 ± 0.001

* data were fitted to a single exponential equation.

Overall, our qualitative and quantitative translocation data show that F95A, although impaired in activation of GTP hydrolysis on the ribosome similarly with D442A, I468A and R472A, is significantly more defective than the other three intrinsically active mutants in mediating the progression of the EF-G cycle from GTP hydrolysis to translocation, being over 90 % less effective and over 450 times slower to translocate than wild type EF-G.

These data suggest that residue F95 is important for mediating the progression of the EF-G cycle from GTP hydrolysis into translocation.

II.2.6. The alanine mutants are fusidic acid resistant

The interface between domains I and III harbors the fusidic acid binding site [6] and the two strongest fusidic acid resistant mutants characterized in *S. aureus* (F95L and H465Y) [15]. In fact, all seven residues we studied in this chapter, except for I468A, are known to harbor spontaneous fusidic acid resistant mutations (F95L, D442N, M461I, L464F, H465Y and R472L, -C, -S, -H

respectively) [17-18]. In order to examine the fusidic acid resistance/sensitivity of our alanine mutants, we examined the linear inhibition of their GTP hydrolysis ability upon titration of increasing concentrations of fusidic acid (Fig. II.11.).

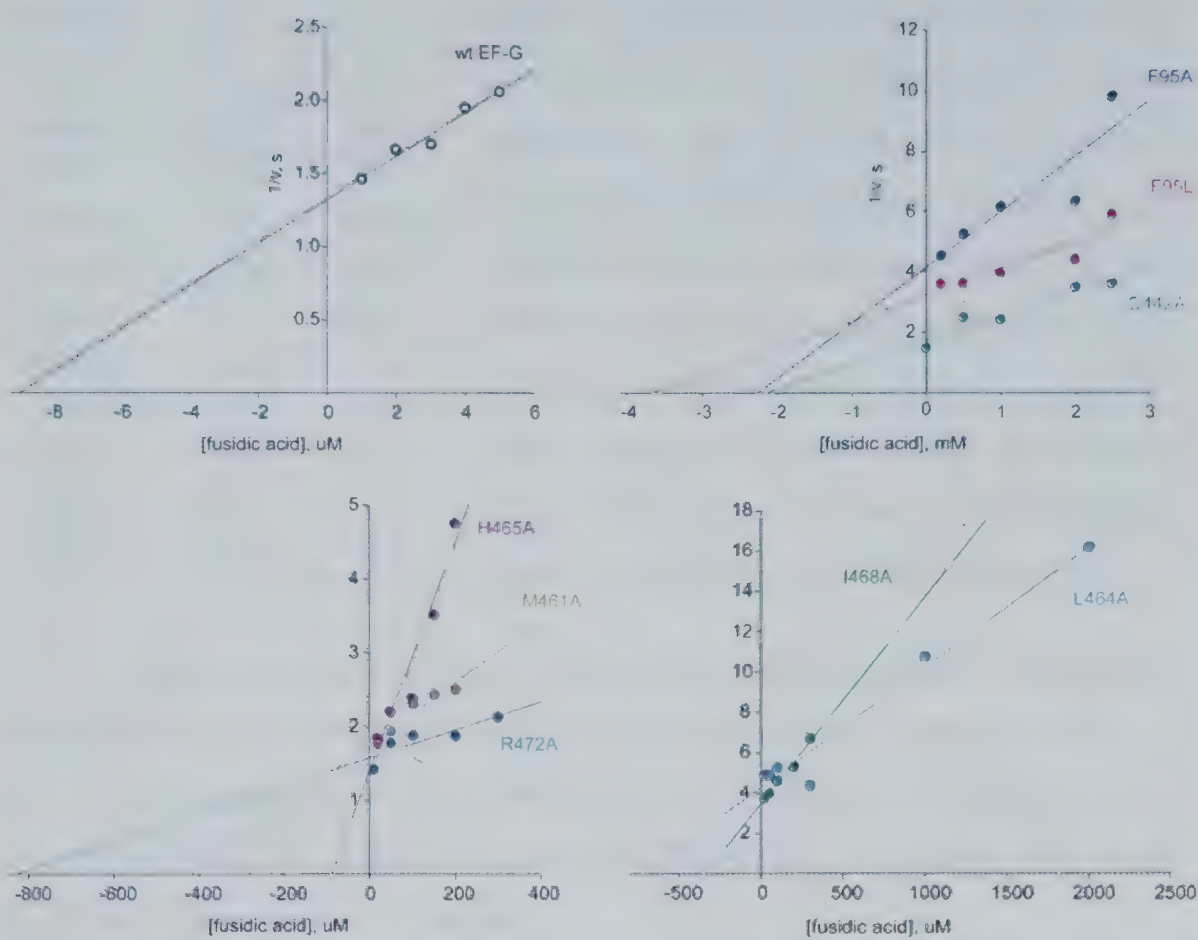


Fig. II.11. Fusidic acid resistance testing
 1 μM 70S, 0.2 μM EF-G, 100 μM GTP and various concentrations of antibiotic were used to determine the sensitivity/resistance of the alanine mutants to fusidic acid. The rates of GTP hydrolysis in the presence of fusidic acid were measured over 1.5 minutes and plotted as above. *Ki* is defined as the minus *x* axis intercept, while the *y* axis intercept is 1/*V*_{max}.

EF-G wild type is well known to be fusidic acid sensitive, while F95L is the strongest fusidic acid resistant mutant identified in *S. aureus* [15]. As expected, F95L showed over 400 fold increased fusidic acid resistance compared with wild type (Table II.5.). Although F95A and D442A show marked fusidic acid resistance (*Ki* in the millimolar range), similar to F95L, due to severe functional impairment, the reported *Ki* values may be affected by the decreased

opportunity for fusidic acid to trap these proteins in a post-translocational ribosomal complex.

Table II.5. Fusidic acid resistance

EF-G mutant	$1/V_{max}$ ($\mu\text{M}^{-1}\text{ s}$)	K_i (μM)
WT	1.33	9
F95L	3.21	3650
F95A	4.14	2250
D442A	2.03	3090
M461A	1.69	317
L464A	4.77	827
H465A	1.31	83
I468A	3.51	342
R472A	1.56	820

Our mutagenesis experiments have created a plethora of fusidic acid resistant mutants (Fig. II.11. and Table II.5.), which is not surprising considering that it has been previously hypothesized [15], and recently proven [6], that the interface between domains I and III of EF-G harbors the fusidic acid binding site. Also, F95L and F95A mutations alter the phenyl ring of F95, which makes direct contact with the antibiotic [6], suggesting the importance of switch II for fusidic acid binding and antibiotic resistance.

Aside from mutation dependent alterations of the antibiotic’s binding site, fusidic acid dependent mutants have also been identified in domains I and V of EF-G [21], suggesting that alternative antibiotic resistance mechanisms may trigger key structural residues within the EF-G molecule, most likely responsible for factor release from the ribosome.

II.3. DISCUSSION

The conserved structural “switch” regions in GTPases (described in [1]) use the energy derived from GTP hydrolysis to irreversibly drive (switch) protein conformations from “on” (active, or GTP state) to “off” (inactive, or GDP state). In the case of EF-G, the “on” state had to be redefined, as kinetic studies showed that GTP hydrolysis happens almost instantly upon EF-G binding to the ribosome, and therefore precedes translocation [30]. As ribosome activated GTP hydrolysis takes place, it is believed to drive synchronous conformational changes of EF-G and the ribosome, which result in Pi release, mRNA and tRNAs translocation, and subsequent release of EF-G and GDP from the ribosome (reviewed in [31]).

Ribosome induced conformational changes of EF-G have been hypothesized to be triggered by GTP hydrolysis and achieved by a “spring” mechanism carried out by the two switch regions in domain I, which unlock conformational rearrangements in EF-G and in turn drive the entire cycle of EF-G on the ribosome [32]. These molecular rearrangements within EF-G are believed to span the G domain all the way to the tip of switch II, through the interface between domains I and III, and further down into domain III [6, 11, 15, 24]. Such “expansion” of the EF-G structure however requires not only great flexibility, but also a certain degree of rigidity, otherwise the tRNAs and mRNA could back-translocate to the A site. Therefore some domains of EF-G must remain anchored to the ribosome, while others rotate and extend with respect to each other. Although identifying the points of flexibility and/or resistance in terms of EF-G structure remains a challenge, most studies to date [15, 18, 21-23, 29, 33-35] agree that domains I and II show less flexibility relative to each other and relative to the ribosome, while the rest of EF-G extends or closes with respect to the interface between domains I/II and III/IV/V.

In order to investigate the role of the interface between domains I and III in terms of progression of the EF-G cycle, we attempted to disrupt the surface contacts between domains by mutating seven well conserved residues from switch II and domain III to alanines (Fig. II.1., D.). Altering the hydrophobic contacts between these two domains could lead to gross conformational changes of the protein, as well as to intrinsic rearrangements of domain I, as reported by crystallographic studies of other mutated residues in this region [15, 18, 29]. In fact, some of the alanine mutants exhibit different fluorescent behavior in the presence of mant-GTP (Fig. II.2. and II.3.), suggesting that the hydrophobicity of the nucleotide binding environment may be altered compared to wild type EF-G.

The alanine mutants showed severe impairment in terms of EF-G cycle progression both in solution and on the ribosome. Their intrinsic GTP hydrolysis activities were up to 30 fold higher than that of wild type EF-G (Fig. II. 4.), without notable alteration in terms of GTP or GDP affinity (Table II.2.). Although bound to the ribosome (Fig. II.5. and II.6.), these mutants’ ability to further

activate GTP hydrolysis is significantly impaired (Fig. II.7. and II.8.). Further progression throughout the EF-G cycle into translocation is also affected (Fig. II.9. and II.10.), especially in the case of F95A, which exhibits 8 % qualitative translocation and over 450 fold impairment in the rate of translocation compared with wild type EF-G.

By examining the structure of EF-G on the ribosome in the GTP state, it appears that the nucleotide is protected by a hydrophobic core formed between switch I, switch II and helix B_{III} residues in domain III [9]. However, in the post-translocational structure of ribosome bound EF-G, switch I flips away from the nucleotide [32], which breaks the interactions with switch II and domains III, while also exposing the γ phosphate of GTP to water attack [6]. Interestingly, domain III deletion studies found that intrinsic GTP hydrolysis is activated by the deletion [24]. It is therefore plausible that our alanine mutations may have indirectly altered the molecular arrangement of domain I in such a way that EF-G is now in a "GTP hydrolysis prone" conformation which normally is only attained in the presence of the ribosome.

Recent crystallographic studies of EF-G on the ribosome propose that residues in domain III contact both ribosomal subunits, "sensing" the ribosome in terms of ratcheted or unratcheted states [6]. In turn, the ribosome, through interactions between EF-G and L7/L12, may also recognize bound EF-G [36]. Also, the interactions between the G' domain and the r-protein L7/L12 [26, 37-38] as well as domain III itself [24] have been shown to be essential for GTP hydrolysis. All these EF-G/ribosome contacts probably "lock" EF-G into place on the ribosome and drive conformational changes within the G domain which activate the GTPase center of EF-G. Thus the defective activation of GTP hydrolysis on the ribosome by some of the alanine mutants could be due to a combination of disrupted EF-G/ribosome contacts as well as deficient inter-domain anchor points, which allow the mutant to bind, but not to be activated by the GAC of the ribosome.

While deficiencies in activation of GTP hydrolysis on the ribosome are comparable between mutants, in terms of translocation impairment, F95A is by

far the most defective. Wild type EF-G (F95) and F95A, although only differing by the length and the degree of hydrophobicity of the side chain, have significantly different functionality, which might suggest that interactions between the phenylalanine ring of F95 and the residues in domain III are primarily responsible for creating the molecular interactions necessary for pivoting domains III, IV and V onto the tip of switch II in order to achieve the “extended” and rotated post-translocational conformation of EF-G [6, 11]. The hypothesis that domain III functions as an intermediate during such movements is supported by the observation that its deletion inhibits translocation despite the presence of an intact domain IV [24].

Visually, the interface between domains I and III of EF-G can be compared to a “ball” (F95) and “socket” (domain III residues) type of articulation. The stability of these interactions may be required in order to lock and/or switch between, certain conformations throughout elongation. It has already been shown that single amino acid mutations in domains I and II can translate into significant conformational changes within the G domain [18]. Thus it is not unreasonable to assume that our mutations may have somehow resulted in an “easily subluxating joint”, by inducing an overly flexible, or unstable, conformation of the protein. In turn, EF-G may be allowed to “slide” between “GTP bound” and “GTP hydrolysis prone” states in solution, which would explain the un-inhibited intrinsic GTP hydrolysis ability of some mutants. Once bound to the ribosome, altered interactions between domain III and the ribosomal subunits may impair activation of GTP hydrolysis, while impaired interactions between switch II and domain III, which maintain the equilibrium between intrinsic rigidity and flexibility, may add to the observed impairment of translocation.

II.4. CONCLUSIONS

In the absence of the ribosome, switch I locks GTP on EF-G [32]. In solution, GTP hydrolysis is prevented, possibly by the very conformation of the

two switch regions EF-G, which protect the nucleotide from water attack [6]. Upon ribosome binding, specific EF-G-ribosome interactions of domain III [6, 24] and domain I [38], may enable conformational rearrangements within EF-G and the ribosome which trigger GTP hydrolysis. Further molecular rearrangements would then propagate from the G domain through the tip of switch II to residues in domain III of EF-G. In conjunction with the ribosome, the inter-domain interface would next allow the rotation and extension of domains IV and V onto domain III [11], thus enabling translocation.

We propose that the interface between domains I and III harbors essential inter-domain contacts which modulate a careful equilibrium between flexibility and rigidity, thus influencing the progression of the EF-G cycle on and off the ribosome. Additionally, these interactions may be important for maintaining the overall intrinsic integrity of EF-G.

II.5. FUTURE DIRECTIONS

This study provides a methodical functional approach for characterizing the role played by interactions between switch II and domain III in terms of EF-G cycle progression. Still, the functional data in this study would be better understood if complemented by structural information on the intrinsic rearrangements triggered by the F95A mutation both in solution and in presence of the ribosome.

Crystal structures of the F95A mutant in the absence of the ribosome may help understand the structural basis for EF-G's ability to inhibit GTP hydrolysis in solution. Shortening of the side chain of F95 upon mutation to alanine, by disrupting interactions with domain III residues (probably D442 and R472 in particular), may cause switch II to adopt an alternative conformation which, possibly combined with further rearrangements of domain I, may activate EF-G's ability to hydrolyze GTP in the absence of the ribosome.

It has been hypothesized that upon binding to the ribosome, domain III interactions with ribosomal protein S12, and helix 5 in the 30S, coordinated with

contacts with the SRL in the 50S, may play a role in triggering GTP hydrolysis [24], by enabling EF-G to “recognize” unratcheted from ratcheted states of the ribosome [6]. It has also been suggested that the ribosome also “proofreads” bound factors by L7/L12 interactions [36]. These bidirectional recognition mechanisms may be further correlated with interactions between the G’ domain of EF-G and the r-protein L7/L12 in terms of full activation of GTP hydrolysis on the ribosome [37-38]. It is therefore possible that some of our alanine mutants, although bound to the ribosome, due to defective contacts with the ribosome triggered by mutational dependent rearrangements of the inter-domain interface, may falsely recognize the ribosome as ratcheted, which would prompt EF-G·GTP to dissociate from the ribosome without activation of GTP hydrolysis.

So far, we do not know whether our mutations have led to rearrangements of just domain III and/or of the G domain itself, as both could affect ribosome interactions and therefore activation of GTP hydrolysis. By comparing the crystal structure of F95A on the ribosome in complex with GDPNP (pre-GTP hydrolysis) with the EF-2·GDPNP structure available [9] (or with a yet to come structure of EF-G·GDPNP), one would be able to clearly identify potentially altered F95A·ribosome interactions, which would in turn indicate which contacts are required for triggering GTP hydrolysis on the ribosome.

II.6. MATERIALS AND METHODS

II.6.1. Reagents, general experimental procedures and buffers

TLC PEI-cellulose plates, DMS, GTP, GDPNP, *E. coli* tRNA^{fMet} and tRNA^{Phe} were purchased from Sigma Aldrich Ltd. The silica gel 60 (F-254) TLC plates were purchased from Brinkman Instruments Canada. The YM-100 500 µl microcons were purchased from Fischer Scientific. [$\gamma^{32}\text{P}$]-GTP (250 µCi), [$\alpha^{32}\text{P}$]-ATP (250 µCi) and [$\alpha^{32}\text{P}$]-TTP (250 µCi) were purchased from Perkin Elmer. Mant labeled GTP and GDP were purchased from Invitrogen Inc. (Molecular Probes). Avian Myeloid Leukemia Virus Reverse Transcriptase (AMV RT) was purchased from Roche Diagnostics and T4 Polynucleotide Kinase (PNK), DNA

ligase, T7 DNA Polymerase (DNAP), T7 RNA Polymerase (RNAP), DNase and *Bam*H1 were purchased from New England Biolabs.

Except where specifically indicated, all experimental reactions in this chapter have been carried out in polymix buffer [28]: 5 mM KH_2PO_4 pH 7.5, 5 mM $\text{Mg}(\text{OAc})_2$, 0.5 mM CaCl_2 , 95 mM KCl, 5 mM NH_4Cl , 1 mM DTT, 8 mM putrescine, 1 mM spermidine. For ease of use, we have created a 10X solution of polymix buffer, which contained all chemical substances except for spermidine, which was prepared separately as a 10 mM (10X) solution. The remaining 10X polymix buffer solution components were combined in the following order: nanopure water (np H_2O), KH_2PO_4 pH 7.5, $\text{Mg}(\text{OAc})_2$, KCl, NH_4Cl , DTT and putrescine. CaCl_2 was added last with vigorous mixing. The 10X polymix buffer solution (without spermidine) was kept at room temperature and re-prepared every two-three months due to calcium compound precipitation over time. The 10 mM (10X) spermidine solution was kept at -20°C .

Other buffers used: **SDS sample buffer**: 30 % (v/v) glycerol, 2 % (v/v) SDS, 20 mM Tris-HCl pH 8.0, 2 mM DTT and 0.025 % bromophenol blue; **urea gel loading buffer**: 7 M urea, 0.025 % (w/v) bromophenol blue, 0.025 % (w/v) xylene cyanol in 2.5 % (v/v) formamide; **buffer 1**: 50 mM Tris-HCl pH 7.5, 1 M NH_4Cl , 10 mM MgCl_2 , 0.5 mM EDTA and 6 mM DTT; **buffer 2**: 50 mM Tris-HCl pH 7.5, 1 M NH_4Cl , 6 mM MgCl_2 and 6 mM DTT; **buffer3**: 50 mM Tris-HCl pH 7.5, 0.1 M NH_4Cl , 10 mM MgCl_2 and 1 mM DTT; **buffer 4**: 1 mM ATP, 50 mM Tris-HCl pH 7.8, 10 mM MgCl_2 , 10 mM DTT and 1 mM spermidine; **buffer 5**: 20 mM Tris-HCl pH 7.4 and 10 mM MgCl_2 ; **buffer 6**: 1 mM dNTPs, 1 mM ATP pH 7.0, 50 mM Tris-HCl pH 7.4, 10 mM MgCl_2 and 5 mM DTT; **buffer 7**: 5 % (v/v) glycerol, 10 mM Tris-HCl pH 8.0, 10 mM MgCl_2 , 0.2 M KCl and 1 mM DTT; **buffer 8**: 5 % (v/v) glycerol, 10 mM Tris-HCl pH 8.0, 10 mM MgCl_2 , 1 M KCl and 1 mM DTT; **buffer 9**: 50 % (v/v) glycerol, 10 mM Tris-HCl pH 8.0, 0.1 M KCl and 3 mM DTT; **buffer 10**: 1 mM ATP, 50 mM Tris-HCl pH 7.8, 10 mM MgCl_2 , 10 mM DTT and 1 mM spermidine; **buffer 11**: 80 mM HEPES-KOH pH 7.7, 12 mM MgCl_2 , 4 mM DTT, 1 mM spermidine and 2 mM of each ATP, GTP, CTP and UTP; **buffer 12**: 100 mM Na_2HPO_4 pH 8.0 and 0.1

mM EDTA; **buffer 13**: 30 mM NaOAc pH 5.2, 1 mM EDTA and 0.1 % (v/v) SDS; **buffer 14**: 70 mM Tris-HCl pH 7.5, 1 mM MgCl₂ and 5 mM DTT; **buffer 15**: 50 mM HEPES-KOH pH 7.7 and 100 mM NH₄Cl; **buffer 16**: 60 mM β -mercaptoethanol, 60 mM EDTA and 1M Tris-HCl pH 7.5; **buffer 17**: 300 mM NaOAc pH 5.2, 0.1 % (v/v) SDS and 1 mM EDTA; **buffer 18**: 50 mM KOH-HEPES pH 7.0 and 100 mM KCl; **buffer 19**: 130 mM Tris-HCl pH 8.5, 10 mM MgCl₂ and 10 mM DTT.

II.6.2. Ribosome purification

E. coli 70S ribosomes were purified from MRE600 cells following the protocol described in [27]. The details of ribosome purification are described below.

MRE600 cells were grown in Luria-Bertani (LB) media up to A_{600nm} of 0.6, harvested by centrifugation (15 minutes at 7,500 x g at 4° C) and stored at - 80°C until further use. From here on, the entire protocol involves working on ice or at 4° C to prevent degradation of the ribosomal particles. 5 g of pelleted cells were thawed on ice, resuspended in ice cold buffer 1 by gentle pipeting and broken using a French Cell Press (at least two pulses of 18,000 p.s.i). The cell debris was separated from the soluble lysate by centrifugation (45 minutes at 30,000 x g at 4° C). The supernatant underwent extensive centrifugation in order to pellet the ribosomes (14 hours at 112,000 x g at 4° C). The crude ribosome extract was then separated from the supernatant and resuspended in 2X (v/v) of buffer 2.

The resuspended extract was then carefully layered on top of a sucrose gradient solution (10-40 % sucrose in buffer 2) and underwent overnight centrifugation in order to separate the ribosome particles from other debris (13 hours at 56,000 x g at 4° C). The 70S particles were separated from 30 and 50S particles by using a Brandel fractionation apparatus, collected and repelleted after adjusting buffer 2's MgCl₂ concentration to 10 mM (20 hours at 73,000 x g at 4° C). The glassy ribosome pellet obtained was separated from the supernatant, resuspended in buffer 3, split into 10-20 μ l aliquots, fast-frozen in liquid nitrogen

and stored at - 80° C until further use. The concentration of the 70S preparation was determined assuming 1 absorbance unit at 260 nm equals 23 µM 70S.

All ribosomes used in experimental assays have been heat activated (5-10 minutes at 37° C) prior to use.

II.6.3. EF-G mutagenesis

E. coli MRE600 *fusA* gene was inserted into the pET24b+ vector (purchased from Novagen) [39]. The kanamycin-resistant pET24-*fusA* plasmid expresses a C terminus hexahistidine tagged *E. coli* EF-G.

The following primers were created: **F95A**: 5'-GTT CTA CTT CGA TTG TTG CGT CAA CGT GCC CCG GGG-3', **F95L**: 5'-GTT CTA CTT CGA TTG TCA GGT CAA CGT GCC CCG GGG-3', **D442A**: 5'-CCA TAC ACG GAA AGA CGG TGC TTC TTT AGC CAG ACG GCC C-3', **M461A**: 5'-CGA GGT GCA GTT CGC CTG CAC CCG CGA TGA TGG TCT GG-3', **L464A**: 5'-CGG TCA ACG ATGATG TCG AGG TGT GCT TCG CCC ATA CCC GC-3', **H465A**: 5'-5'-CGG TCA ACG ATGATG TCG AGT GCC AGT TCG CCC ATA CCC GC- 3', **I468A**: 5'-CGT TGA ATT CAC GCT TCA TAC GGT CAA CGA TTG CGT CGA GGT GCA GTT CGC CCA TAC CCG-3', **R472A**: 5'-CGT TGA ATT CAC GCT TCA TTG CGT CAA CGA TGA TGT CG-3'.

These mutations were introduced into the pET24-*fusA* gene by using site-directed mutagenesis [40]. 100 pmols of primer were incubated for 45 minutes at 37° C with 1 U T4 PNK in buffer 4. 12 pmols of phosphorylated primer were incubated with 0.5 pmols of ssDNA (from *ung*⁻ *dut*⁻ strain) for four minutes at 90° C in buffer 5, immediately followed by 20 minutes incubation at 37° C (41° C for D442A and R472A). Reactions were then transferred to ice for five minutes. The annealed primers were then incubated for 90 minutes at 37° C with 10 U of DNA Ligase and 3 U of T7 DNAP in buffer 6. The DNA was then precipitated (1/10X (v/v) 3 M NaOAc pH 5.2 and 3X (v/v) 100 % ethanol at - 20° C) overnight. The pelleted DNA was then transformed by electroporation into DH5α cells, incubated for 45 minutes at 37° C, and then plated on 30 µg/ml kanamycin LB agar plates overnight at 37° C.

The DH5 α colonies were screened by mini-plasmid prep protocols (Qiagen) followed by DNA sequencing and cross-matching with wt using the BioEdit software. The chosen mutated plasmids were then introduced by electroporation into *E. coli* BL21(DE3) cells, incubated for 45 minutes at 37° C and plated onto 30 μ g/ml kanamycin LB agar plates overnight at 37° C.

BL21 (DE3) colonies were grown in LB media with 30 μ g/ml kanamycin at 37° C until $A_{600nm} \approx 0.7$, then the over-expression of the hexahistidine tagged EF-G was induced with 1 mM IPTG. After an additional four hours at 37° C, cells were harvested by centrifugation (15 minutes at 7,500 x g at 4° C) and pellets were stored at - 80° C until further use.

II.6.4. EF-G purification

Approximately 10 g of BL21(DE3) pelleted *E.coli* cells were submitted to two rounds of purification: first, Ni-NTA purification isolated the hexahistidine protein from the *E. coli* lysate [27], then size exclusion chromatography identified and isolated the properly folded, non-aggregated protein species.

II.6.4.1. Ni-NTA purification: Cells were lysed on ice for 10 minutes using 10 % (v/v) BugBuster solution, containing Protease Inhibitor Cocktail, followed by a brief centrifugation step (25 minutes at 9,500 x g at 4° C) which separated the cell debris from the soluble lysate. From here on, the entire protocol involves working at 4° C to minimize proteolytic activity. The supernatant was applied on top of 3 ml Ni-NTA agarose beads poured into a single use column and equilibrated in buffer 7. The column was left to drain by gravity (1 hour) at 4° C.

The column was then connected to a BioRad Biologic LP apparatus, washed with 8 CV (column volumes) of buffer 7, followed by an additional 8 CV of buffer 8 in order to eliminate the unbound (non-histagged) proteins. The hexahistidine tagged protein was eluted under an imidazole gradient (200 μ M to 1M) in buffer 7. EF-G eluted between 250-350 mM imidazole.

Aliquots of the corresponding elution fractions were denatured for 10 minutes at 90° C in SDS sample buffer and analyzed by 10-12 % SDS-PAGE (Fig. II.12).

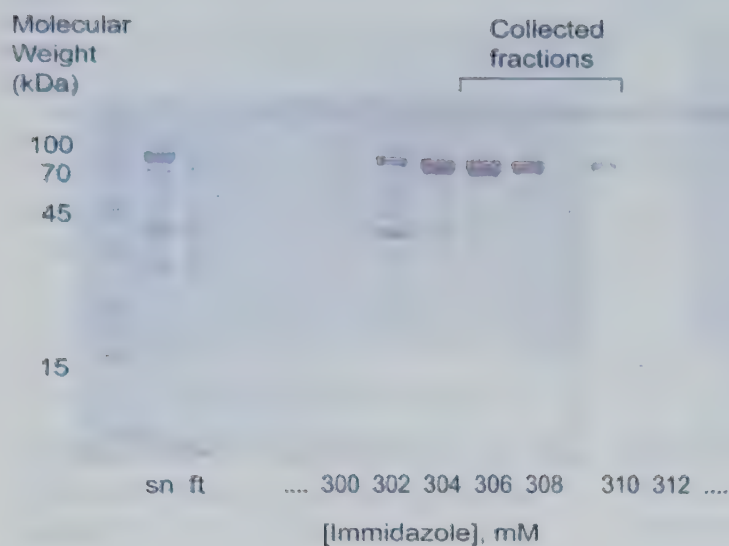


Fig. II.12. Ni-NTA purification of EF-G (example of wild type)
The supernatant (sn) resulting from the cell lysate was applied onto the Ni-NTA and let drain by gravity. The flow through (ft) shows over 90 % binding of EF-G to the Ni-NTA column.

The purest fractions were pooled together, dialyzed (35 kDa cut-off point) overnight at 4° C against buffer 7 to eliminate the imidazole, and submitted to further purification steps.

II.6.4.2. Size exclusion chromatography purification: The dialysis product was loaded onto a Superdex 200 (10/300) size exclusion chromatography column (Amersham Biosciences), equilibrated in buffer 7 and connected to an ÄKTA purifier apparatus (Amersham Biosciences). The peak profile was recorded and analyzed using the Unicorn software, showing only minimal contamination (less than 1 %) of the major EF-G product.

Aliquots of the corresponding elution fractions were denatured for 10 minutes at 90°C in SDS sample buffer and analyzed by 10-12 % SDS-PAGE (Fig. II.13). The purest fractions were pooled together, dialyzed overnight at 4°C against buffer 9 and stored at – 20 °C until further use.

The estimated quality of the final protein stock is over 97 % purity (Fig. II.14). The EF-G concentration was determined by $A_{280\text{nm}}$ using an extinction coefficient of $64,282 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

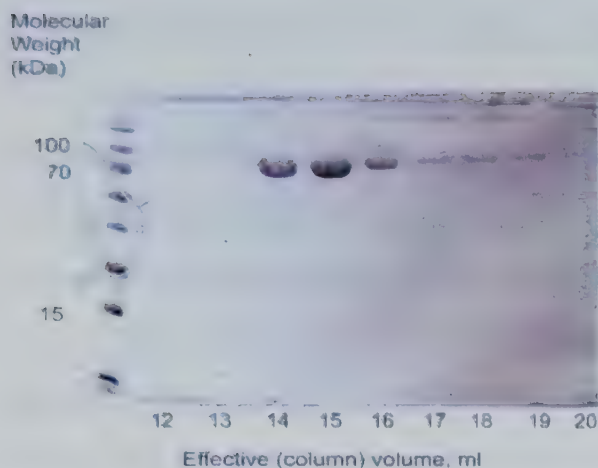


Fig. II.13. EF-G purification by size exclusion chromatography column (example of wild type).

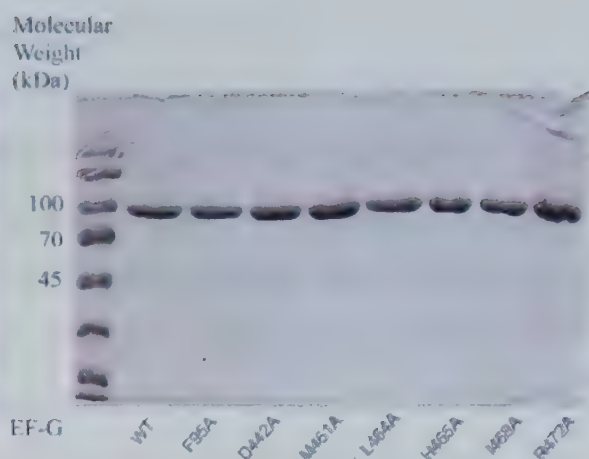


Fig. II.14. Result of double column purification of EF-G
20 pmols of the final stocks of wild type and alanine mutants.

II.6.5. mRNA purification

The phage T4 gene 32 mRNA fragment (128 nucleotides) used in the toeprinting experiment was cloned by Rachel Green, Johns Hopkins University, between the *EcoRI* and *BamHI* sites of pUC118.

The plasmid was cut at 37° C with 1 µg *BamHI* per 1 µl plasmid in buffer 10. The reaction was stopped after 4 hours and the DNA was precipitated overnight. The efficiency of *BamHI* digestion of the plasmid was confirmed by 1 % agarose with 0.5 % ethidium bromide gel.

The cut plasmid was transcribed by 1,250 U (40X excess) T7 RNAP to digested plasmid in buffer 11 for 2 hours at 37° C. The transcribed RNA was further purified by one phenol-chloroform extraction (1:1 v/v) and precipitated overnight at - 20° C.

The precipitated RNA was separated in nanopure water from unincorporated NTPs by size exclusion chromatography using a Superdex 200 (10/300) column connected to an ÄKTA purifier and analyzed using the Unicorn software. The mRNA peak was collected and purified by overnight precipitation.

The mRNA was stored in np H₂O at - 20° C and its concentration determined using an extinction coefficient of 25 mg⁻¹ · ml⁻¹ · cm⁻¹.

II.6.6. Pyrene-labeled mRNA oligonucleotide purification

The mRNA oligonucleotide (5'-AAGGAGGUAAAAAUGUUUGCUN₆-3') was synthesized by Dharmacon and labeled with pyrene as described [26, 28]. The RNA fragment and DMS-solubilized pyrene butyryl hydroxysuccinimid (1:1 v/v) were incubated for 4 hours at 60° C (with hourly gentle mixing) in buffer 12.

The RNA was then precipitated overnight. The pelleted RNA was resuspended in urea gel loading buffer and separated by 7 M urea 10 % PAGE for 4 hours at 35 W. The pyrene labeled RNA fragment was visualized under 254 mM ultraviolet light (UV) using an Upland UV lamp, model UVLS-24. The pyrene-mRNA oligonucleotide was then cut from the gel with a sterile (RNase free) scalpel blade, resuspended in 2X (v/v) buffer 13 and eluted overnight under gentle rotation. The eluted RNA was then precipitated overnight at - 20° C and resuspended in np H₂O.

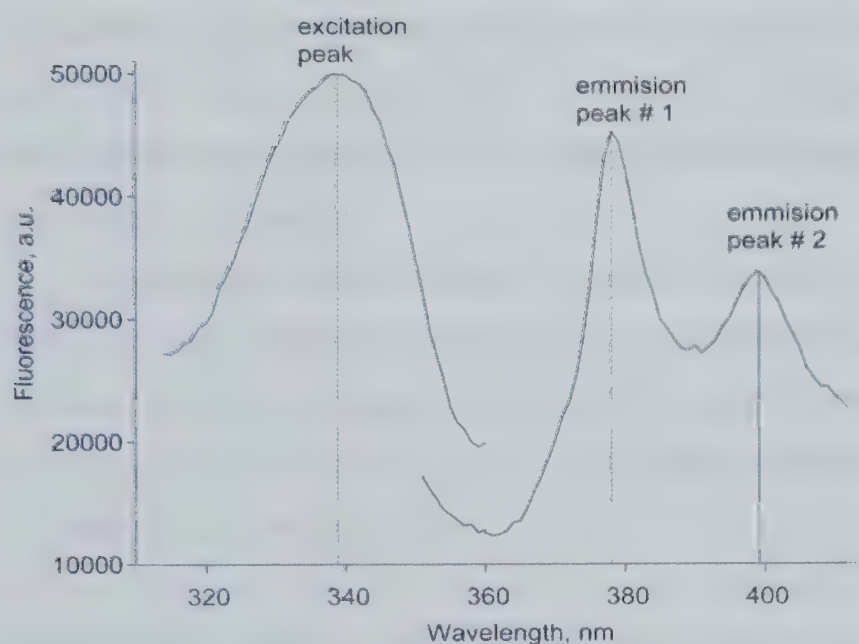


Fig. II.15: Pyrene group fluorescence excitation and emission spectra

The presence of the pyrene group was demonstrated by looking at the fluorescence spectrum (Fig. II.15) of the resultant sample (excitation peak at 335 nm and emission peaks at 378 nm and 398 nm respectively). Its concentration was determined by absorbance using an extinction coefficient of $0.2257 \text{ mg}^{-1} \cdot \text{ml}^{-1} \cdot \text{cm}^{-1}$.

II.6.7. GTP hydrolysis assay

Multiple round GTP hydrolysis reactions (20 μ l final volume) were carried out at room temperature, by mixing equal volumes of 200 μ M GTP (with 0.05 μ Ci [γ ³²P]-GTP) and 0.8 μ M EF-G pre-incubated (5 minutes at 37° C) with, where applicable, with increasing concentrations of 70S ribosomes.

The reactions were stopped by quenching 2 μ l aliquots taken at various time intervals with 2 μ l 30 % formic acid. Samples were then separated in 3.5 M formic acid pH 4.0, using water pre-run TLC PEI-cellulose plates. The plates were then washed in acetone, dried and quantified after overnight exposure to Phosphorimager plaques by using ImageQuant software.

Data analysis was done using Sigmaplot 11 and Microsoft Excel 2007 software. All EF-G intrinsic GTP hydrolysis values were normalized with respect to the residual hydrolyzed GTP control in each reaction (values ranging between 0.01 and 0.03), while ribosome activated values of GTP hydrolysis were normalized with respect to the intrinsic GTP hydrolysis ability of the ribosome prep itself (values between 0.03 and 0.04). Individual rates (k_{app}), were determined using a minimum of four time points falling in the linear range of the curve (up to 2 minutes).

Determination of ribosome dependent K_M (μ M) and k_{cat} (s^{-1}) for GTP hydrolysis was calculated by plotting the corresponding k_{app} values as a function of 70S concentration (ranging from 0.0625 to 2 μ M). The equation used was $v = v_o + (k_{cat} \cdot [EF-G] \cdot k_{app}) / (K_M + k_{app})$. All reported results are an average of three or more independent experiments.

II.6.8. Fusidic acid K_i determination using the GTP hydrolysis assay

Multiple round GTP hydrolysis reactions (20 μ l final volume) were carried out at room temperature, by mixing equal volumes of 200 μ M GTP (with 0.05 μ Ci [γ ³²P]-GTP) and 0.4 μ M EF-G pre-incubated (5 minutes at 37° C) with 2 μ M 70S and various concentrations of fusidic acid. 2 μ l aliquots were extracted at 10, 30, 45, 60 and 90 seconds, quenched and separated as described, followed by ImageQuant software analysis.

We observed that at high enough fusidic acid concentrations, GTP hydrolysis was inhibited below the intrinsic 70S values; therefore the fusidic acid inhibited GTP hydrolysis values were all normalized with respect to the constant value of residual hydrolyzed GTP control (0.01 to 0.02). For the determination of K_i (μM), the values of fusidic acid dependent k_{app} (s^{-1}) were plotted as $1/k_{app}$ as a function of fusidic acid concentration. $1/V_{max}$ was defined as the y axis intercept, whereas K_i was defined as the $-x$ axis intercept.

II.6.9. Mant-GTP and mant-GDP binding

Fluorescence experiments were performed using a PTI QM-6 (Photon Technology International) equipped with a LPS-220B lamp. Kinetic experiments used a stopped-flow MiniMixer (KinTek Corporation) device attached to the PTI QM-6 fluorimeter, which acts as to rapidly mix two samples of roughly 180 μl each.

To measure the mant-GTP binding affinity to EF-G, the fluorescence of the mant group was excited by FRET (Fluorescent Resonance Energy Transfer) from tryptophan at 290 nm and measured at 438 nm as described [25]. Reactions (80 μl) were carried out at room temperature in a QT-120 quartz cuvette containing 1 μM EF-G. Increasing amounts of mant-GDP/GTP were added and, after subtracting the control values of mant-GTP/GDP titration over buffer, the stable levels of fluorescence attained in presence of EF-G were plotted (using Sigmaplot 11 software) as a function of mant-nucleotide concentration to determine k_{d-app} (μM) of EF-G for mant-GTP/GDP. The equation used was $v = v_0 + V_{max}/(k_{d-app} + [\text{mant-GTP/GDP}])$. The results are an average of three or more independent experiments.

The fluorescence signal for mant-GTP binding had different behavior than wt for F95A, D442A, M461A, I468A and R472A. To measure the kinetics of mant-GTP binding, the stopped-flow device was used to rapidly mix 1 μM EF-G with increasing concentrations of mant-GTP. The k_{app} (s^{-1}) was calculated from the linear part of the curve and used to determine k_{d-app} (s^{-1}) for mant-GTP binding

using the same equation as above. The results are an average of at least four stopped-flow shots.

II.6.10. EF-G mant-GTP binding dependent fluorescence changes analysis

The fluorescence of the mant group was excited at 362 nm and measured at 445 nm at room temperature using the same PTI QM-6 fluorimeter apparatus as above.

Reactions (80 μ l) started with 120 μ M mant-GTP, then 25 μ M EF-G was added and sample 1 (S1 - 20 μ l) was removed. Next, 1.4 μ M 70S was added and sample 2 (S2 - 20 μ l) was removed. Upon removal, samples were immediately quenched by addition of 20 μ l of 0.2 M HCl. Samples were then concentrated to roughly 5 μ l volume by centrifugation under vacuum (approximately 30 minutes), loaded onto methanol-pre-treated silica gel 60 (F-254) TLC plates and separated in 9:5:1 (v/v/v) 2-propanol : 25 % ammonium hydroxide (v/v) : nanopure water as described [41]. Loading controls were 500 pmols of each mant-GTP and mant-GDP, spotted directly onto the silica gel 60 (F-254) TLC plates.

Separated mant nucleotides were visualized under UV light (365 nm), photographed and imported into Photoshop software.

II.6.11. Toeprinting single round translocation assay

The toeprinting method described in [37], with certain modifications, was used to determine the extent of translocation in the presence of the alanine mutants compared with wild type EF-G. These experiments used our 128 nucleotide long mRNA fragment from phage T4 gene 32.

In order to label the DNA mRNA primer with P^{32} , 1 μ M DNA primer (5'-TTT ATC TTC AGA AGA AAA AC-3') was incubated with 1/10 (v/v) [$\gamma^{32}P$]-ATP and 1.4 U T4 PNK for 1 hour at 37° C in buffer 14. The phosphorylation reaction was quenched using 45 mM EDTA, followed by one phenol chloroform extraction and overnight precipitation, using 1 μ g/ μ l glycogen as carrier. The pelleted ^{32}P labeled primer was then annealed in a ratio of 2:1 with mRNA for 3 minutes at 60° C in buffer 15. The $MgCl_2$ concentration was then adjusted to 10

mM and 2:1 μM primer:mRNA complex was mixed with 1 μM 70S ribosomes (5 minutes at 37° C), followed by addition of 1.25 μM tRNA^{fMet} (30 minutes at 37° C) and 1.25 μM tRNA^{Phe} (20 minutes at 37° C).

Formed ribosomal complexes were then transferred to ice for 5 minutes. 1.25 μM EF-G was then added together with 2 mM GTP and the reactions were left to progress for 10 minutes at 37° C and then transferred to ice for 5 minutes. The annealed DNA primer was then extended for 5 minutes at 37° C using 3 U of AMV RT and 1mM dNTPs. The extension reaction was quenched by denaturation for 3 minutes at 90° C in 2X (v/v) urea gel loading buffer. The samples were then separated on a 7 M urea 6 % PAGE for 2.5 hours at 35 W.

The gel was dried, exposed to a phosphorimager plate overnight and the extent of translocation was quantified using ImageQuant software. The post-translocational band was quantified with respect to both pre-translocational bands. The final extent of translocation values were obtained by normalization with respect to the wild type EF-G value (arbitrarily set to equal 1).

II.6.12. Fluorescence single round translocation assay

Ribosomal complexes were formed as described [26, 28]. Complexes were formed at 37° C and contained 0.4 μM pyrene-labeled RNA nucleotide, 0.5 μM 70S ribosomes (6 minutes incubation), 0.6 μM tRNA^{fMet} in the P site (30 minutes incubation) and 0.6 μM tRNA^{Phe} in the A site (20 minutes incubation).

The same PTI QM-6 fluorimeter and MiniMixer stopped-flow apparatus (see section II.4.8.) were used to rapidly mix at room temperature equal volumes of pre-formed ribosomal complexes and 2.5 μM EF-G with 2 mM GTP. The pyrene fluorescence changes upon translocation were recorded by exciting pyrene at 335 nm and detecting its emission at 378 nm.

The data were analyzed with Sigmaplot 11 software using the equation $F_t = F_0 + \Delta F_1 \cdot \exp(-k_1 \cdot t) + \Delta F_2 \cdot \exp(-k_2 \cdot t)$, where F_t is the fluorescence value (a.u.) at time t (s), F_0 is the fluorescence value (a.u.) at time 0, ΔF_1 and ΔF_2 are the amplitude of the fluorescence changes (a.u.), while k_1 and k_2 represent the

respective rates (s^{-1}) of the two exponential decays. All results are an average of four or more stop-flow shots.

II.6.13. Ribosome-binding experiments

Reactions (20 μ l) containing 1 mM GDPNP, 1 μ M EF-G and 1 μ M of 70S ribosomes, were incubated for 20 minutes at 37° C.

II.6.13.1. DMS modification: the rRNA was alkylated [27, 42] for 10 minutes at room temperature by incubation with 35 mM DMS in 100 % ethanol. DMS was quenched with buffer 16. The RNA was precipitated for 30 minutes. Samples were then centrifuged for 15 minutes at 13,800 x g, pellets were resuspended in buffer 17 and extracted six times with phenol:chloroform (1:1 ratio v/v). Protein free RNA was precipitated again for 30 minutes. Samples were then centrifuged for 25 minutes at 13,800 x g and pellets were resuspended in 125 μ l np H₂O. RNA samples were stored at - 80° C until further use.

Primer extension reactions were carried out as described [27, 42], with only minor changes: 0.2 μ M DNA primer (3'-AAG TTT CGT GCT TAG AT-5') was annealed for 1 minute at 90° C to 0.08 μ M RNA sample in buffer 18. The samples were then let cool down over 10 minutes to 46° C. The primer was first extended for 30 minutes at 37° C using 1U AMV RT, 36 μ M dNTPs (except for dTTP, which was composed of 0.005 μ M [α^{32} P]-TTP and 2 μ M dTTP) in buffer 19. Then 15 mM dNTPs were added to the reaction and the extension process was allowed to proceed for another 15 minutes at 37° C. The reactions were quenched by precipitation for 10 minutes at room temperature. Samples were then spun for 15 minutes at 13,800 x g, the pellets were resuspended in 10 μ l urea gel loading buffer and denatured for 3 minutes at 90° C. 1 μ l from the denatured samples was loaded on a 7 M urea 6 % PAGE and run at 60 W for 4 hours. The gel was dried under vacuum and exposed overnight to a Phosphorimager plate.

The bands corresponding to A2660 modifications were quantified using ImageQuant software followed by Microsoft Excell analysis. The maximal peak values were corrected with respect to the DMS modification control (DMS modification band) and expressed as percentual values of modification of A2660.

The unmodified percentage was considered to reflect the opposite of DMS modification, EF-G binding. Then the value determined for wt binding was arbitrarily set to equal 1 and the rest of the mutants expressed as percentage of binding with respect to the wild type EF-G protein.

II.6.13.2. Filtration [27]: the complexes were diluted in 1 ml of ice cold polymix buffer with 1 mM GDPNP, separated into two samples and filtered individually in pre-treated 500 μ l YM-100 (100,000 Da exclusion limit) microcons by centrifugation, for 5 minutes at 13,800 x g. The two corresponding 25 μ l samples were then pooled together, reduced to 20 μ l by centrifugation for 15 minutes under vacuum, denatured for 10 minutes at 90° C in SDS loading buffer and analyzed by 10-12 % SDS-PAGE gel.

YM-100 microcon pre-treatment: microcons were left to sit on the bench in 200 μ l 0.01 % (v/v) Tween-20 in np H₂O and subsequently spun for 2 minutes at 13,200 x g. The microcons were then washed in 500 μ l np H₂O and spun for 5 minutes at 13,200 x g (step repeated twice). Finally, microcons were equilibrated by adding 500 μ l reaction buffer with 1 mM GDPNP and spun for 5 minutes at 13,200 x g (step repeated twice).

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III. SWITCH I OF EF-G FLIPS OUT OF THE RIBOSOMAL CAVITY UPON GTP HYDROLYSIS AND 30S TRANSLOCATION, THUS FACILITATING GDP RELEASE AND EF-G RECYCLING

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III.1. INTRODUCTION

As discussed in chapter II, the two GTPases involved in bacterial elongation, EF-G and EF-Tu, use ribosome activated GTP hydrolysis to unlock conformational changes of two conserved “switch” elements (switch I and switch II), which further propagate through the GTPase molecule to activate the specific functions of EF-G and EF-Tu on the ribosome [1].

Crystallographic studies of free EF-Tu [2], as well as in complex with GDPNP [3] and GDP [4], suggest that the conformational changes of switch I and switch II of EF-Tu act in cooperation to open a cleft between domains I and II [5], which culminates with the release of the aa-tRNA into the 30S A site and the dissociation of the EF-Tu·GDP complex from the ribosome [6]. As discussed in detail in chapter II, similar GTP hydrolysis activated structural rearrangements occur in EF-G and “unlock” its cycle on the ribosome [7-9], thus allowing translocation and Pi release [10].

In EF-G, switch II may mediate inter-domain interactions which stabilize the rotation of domains I and II with respect to domains III, IV and V (chapter II) during translocation. Unlike switch II, which appears ordered, the switch I element appears either completely or partially disordered in most structural studies to date. Indeed, neither structures of EF-G crystallized in solution with GDP [11-14] or GDPNP [15], or of its eukaryotic homolog, eEF-2, crystallized in the presence or absence of sordarin [16], nor cryo-EM maps of EF-2 bound to the 80S ribosome before and after GTP hydrolysis [17] or before translocation [18], have been able to visualize an ordered switch I region. Still, a recent study combining crystallographic data of *T. thermophilus* apo EF-G-2·GDPNP with a cryo-EM map of ribosome bound *E. coli* EF-G·GDP, was able to identify a completely ordered switch I, situated adjacent to the γ phosphate of the GTP analog, GDPNP [19]. By contrast, a very recent, high resolution (3.6 Å), crystal structure of EF-G bound to the ribosome in complex with fusidic acid, once again finds switch I distorted, but appearing to have moved away from the nucleotide bound to EF-G, GDP [20].

In light of the existing literature supporting significant conformational changes of EF-G throughout its cycle on and off the ribosome, as well as in light of our own unpublished results (see chapter II), we wondered at which point in the EF-G cycle does switch I become distorted and, most importantly, where is it moving to? By clarifying these questions one could establish the role of switch I movement and therefore gain further insight into EF-G function.

In this study, we identified six functional states of EF-G on and off the ribosome and investigated the functional and structural role of the switch I region of EF-G. We observed that in solution and on the pre-translocational ribosome, switch I is tucked in close to GTP, probably protecting the γ phosphate from water attack [20], while in the post-translocational EF-G ribosomal complex, switch I oscillates between flipped-in and flipped-out conformations.

III.2. RESULTS

III.2.1. Defining six functional states of EF-G, free in solution and in complex with the ribosome

In order to investigate the EF-G cycle, we started by defining three EF-G states in the absence of the ribosome and three EF-G states in the presence of the ribosome (Fig. III.1.).

After being released from the ribosome in complex with GDP (named the “GDP(free)” state), EF-G releases the nucleotide and passes through an intermediate nucleotide-less state, “(free)”, only to rapidly bind GTP and re-enter the elongation cycle as EF-G “GTP(free)”.

On the ribosome, we trapped EF-G in three additional states, named “GDPNP”, “vio” and “fus”. GDPNP is a non-hydrolysable GTP analog, while viomycin and fusidic acid are antibiotics. Viomycin is a translocation inhibitor, believed to bind to the ribosome at the inter-subunit interface, near the 30S A site [21]. It allows for GTP hydrolysis by EF-G and for ribosomal 50S translocation, but prevents 30S translocation [22]. As discussed in detail in chapter II, fusidic acid traps EF-G·GDP on the ribosome in a post-GTP hydrolysis post-

translocational state. Additionally, throughout this chapter, the EF-G-ribosome complex in presence of GTP or GDP only will be referred to as the “GTP” or “GDP” states, respectively.

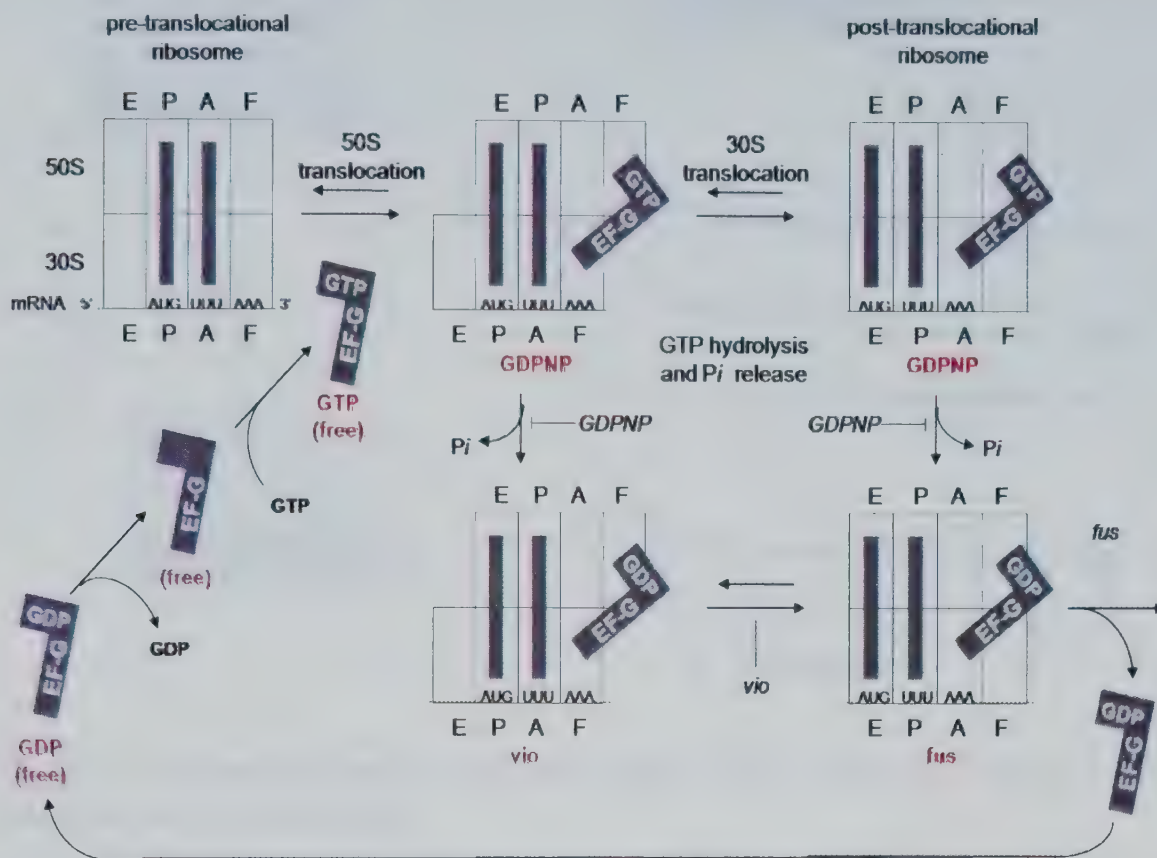


Fig. III.1. The EF-G catalytic cycle

The individual boxes represent the 50S and 30S A (aminoacyl), P (peptidyl), E (exit) and F (factor, e.g. EF-G) ribosomal sites. Subunit ratcheting is shown by shifts in the 50S and 30S box rows with respect to each other. The EF-G states are marked in red. The nucleotide bound to EF-G in various states is as marked, except for the GDPNP state, where the EF-G is trapped on the ribosome in a GTP-like state by the non-hydrolysable GTP analog, GDPNP.

In order to better understand the ribosome bound states of EF-G, we proceeded to characterize them in terms of GTP hydrolysis and translocation (Fig. III.2.). All complexes were formed by adding the inhibitor together with EF-G to the ribosome, except for viomycin, which was pre-incubated with the ribosome to allow for the formation of the ribosomal ratcheted state formation before EF-G and GTP addition.

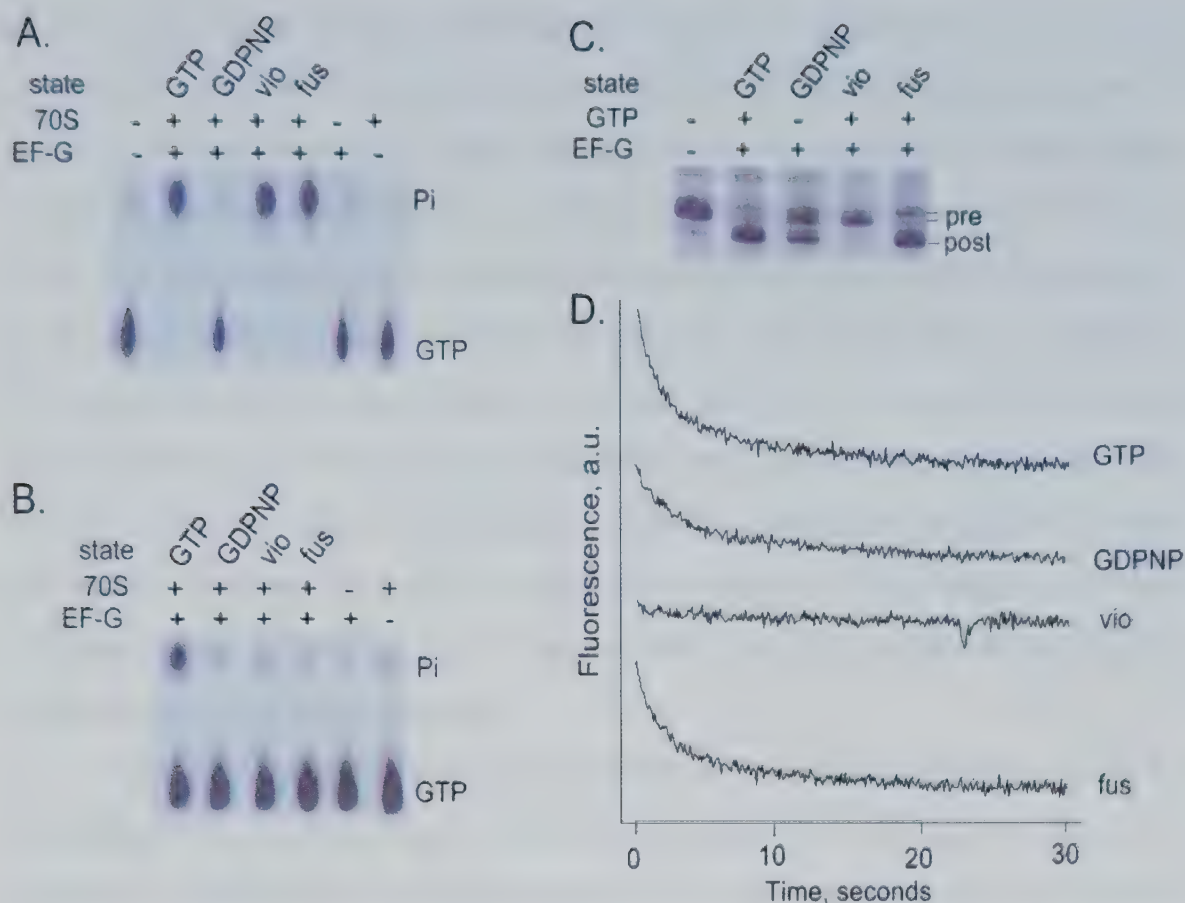


Fig. III.2. Characterizing the ribosome bound EF-G states in terms of GTP hydrolysis and translocation

A., B. GTP hydrolysis. (A) Single turnover conditions: 1 μM 70S, 0.8 μM EF-G, 0.5 μM GTP (with trace $[\gamma^{32}\text{P}]\text{-GTP}$). (B) Multiple-turnover conditions: 0.5 μM 70S, 0.04 μM EF-G, 50 μM GTP (with trace $[\gamma^{32}\text{P}]\text{-GTP}$). In both panels A. and B., the complexes were probed in presence of 0.1 mM GDPNP, 0.25 mM viomycin and 0.2 mM fusidic acid, quenched with 30 % formic acid and then separated by TLC.

C., D. Translocation. (C) Toeprinting: pre-translocational ribosomal complexes were assembled with pre-annealed 1:0.5 μM primer:mRNA, and 1 μM 70S, 1.2 μM tRNA^{fMet} (P site) and 1.2 μM tRNA^{Phe} (A site). (D) Fluorescence-based pyrene-mRNA translocation: pre-translocational ribosomal complexes were assembled with 0.4 μM pyrene labeled mRNA oligonucleotide, 0.5 μM 70S, 0.6 μM tRNA^{fMet} (P site) and 0.6 μM tRNA^{Phe} (A site). In both panels C. and D., the pre-translocational complexes were probed in presence of 1 μM EF-G and 1 mM GTP (“GTP”), 1 mM GDPNP (“GDPNP”), 0.5 mM viomycin and 1 mM GTP (“vio”), and 1 mM fusidic acid and 1 mM GTP (“fus”) respectively.

Data were obtained by Boray Nguyen (panels A. and B.) and Roxana Nechifor (panel D.), and modified from [23].

Under single turnover conditions (Fig. III.2., A.), ribosome activated GTP hydrolysis by EF-G was completely inhibited by GDPNP and unaffected by fusidic acid and viomycin. Under multiple turnover conditions however, both antibiotics showed similar degrees of GTP hydrolysis inhibition as GDPNP (Fig. III.2., B.). Although it was initially believed that fusidic acid is able to trap EF-G in complex with GDP on the ribosome after the first round of GTP hydrolysis [24], recent smFRET experiments show that EF-G is in fact able to undergo several rounds of GTP hydrolysis before being irreversibly trapped by fusidic acid on the ribosome [25]. Our multiple turnover conditions experiment cannot distinguish between the two possibilities. However we conclude that both antibiotics trap EF-G on the ribosome after the first molecule of GTP is hydrolyzed or very early thereafter.

Both our translocation assays, qualitative, toeprinting [26] (Fig. III.2., C.) and quantitative, fluorescence based [27-28] (Fig. III.2., D.), agree that EF-G catalyzed rapid and efficient translocation on the ribosome in its physiological (GTP) state. Also, both assays support our previous finding that in the GDPNP state, EF-G is able to catalyze partial translocation [26]. These results are in agreement with [29], however our approximately three fold slower kinetics of translocation in the GDPNP compared with the GTP state, is a considerably smaller effect than reported by [30-31]. Viomycin showed complete inhibition of translocation in both assays, while fusidic acid slightly inhibited the extent, but not the rate of, translocation (Fig. III.2., C., D.).

From these data we conclude that GDPNP traps the EF-G-ribosome complex in a pre-GTP hydrolysis and mostly post-translocational state [9, 26, 32], viomycin, in a post-GTP hydrolysis pre-translocational state [22], while fusidic acid traps a post-GTP hydrolysis post-translocational complex [20].

III.2.2. The GDP states of EF-G are more accessible to trypsin cleavage than the GTP states

In order to probe EF-G's conformation in solution and on the ribosome, we started by monitoring the cleavage pattern related to trypsin exposure of EF-G

in all our previously defined states. Upon trypsin exposure, we observed a ubiquitous, readily detectable 72 kDa peptide fragment, which had been observed by a previous study of free EF-G trypsin probing [33]. Other smaller peptide fragments only appeared after the majority of intact EF-G had been digested.

In the absence of the ribosome, the 72 kDa fragment was formed slowest in the GTP(free) state and fastest in the (free) state (Fig. III.3., A.). The GDP(free) state appeared to have an intermediate time dependent cleavage pattern of EF-G compared with the other two free EF-G states.

In order to study the remaining three ribosome bound states, we formed the pre-translocational ribosomal complexes with substoichiometric amounts of EF-G relative to the ribosome, and purified them away from free EF-G by filtration (Fig. 3.III., B.). Upon trypsin digestion of the purified complexes, the 72 kDa peptide fragment was detected to be most abundant in the vio state and least abundant in the GDPNP state. In order to establish whether trypsin indeed digested ribosome bound EF-G rather than post-filtration dissociated protein, we further purified the digested complexes by a second filtration step. We found that the GDPNP state did not contain cleaved EF-G, whereas the fus state sample appeared to contain mostly cleaved protein. The vio state showed no detectable EF-G, intact or cleaved, suggesting that EF-G had dissociated from the ribosome in the process.

These results indicate that the vio complex is not as stable as the other two ribosome bound states. We therefore turned our attention to the more stable GDPNP and fus complexes, and probed them with trypsin over time (Fig. 3.III., C.), as we had done for the free EF-G states (Fig. 3.III., A.). We found that the trypsin cleavage 72 kDa fragment was formed significantly slower in the GDPNP state compared with the fus state.

To summarize our trypsin probing experiments, we have observed a higher susceptibility of EF-G to trypsin attack in the GDP(free), (free) and fus states compared with the GTP(free) and GDPNP states. More concisely, the states containing GDP rather than GTP (or GTP analog) bound to EF-G appear to be more exposed to trypsin attack.

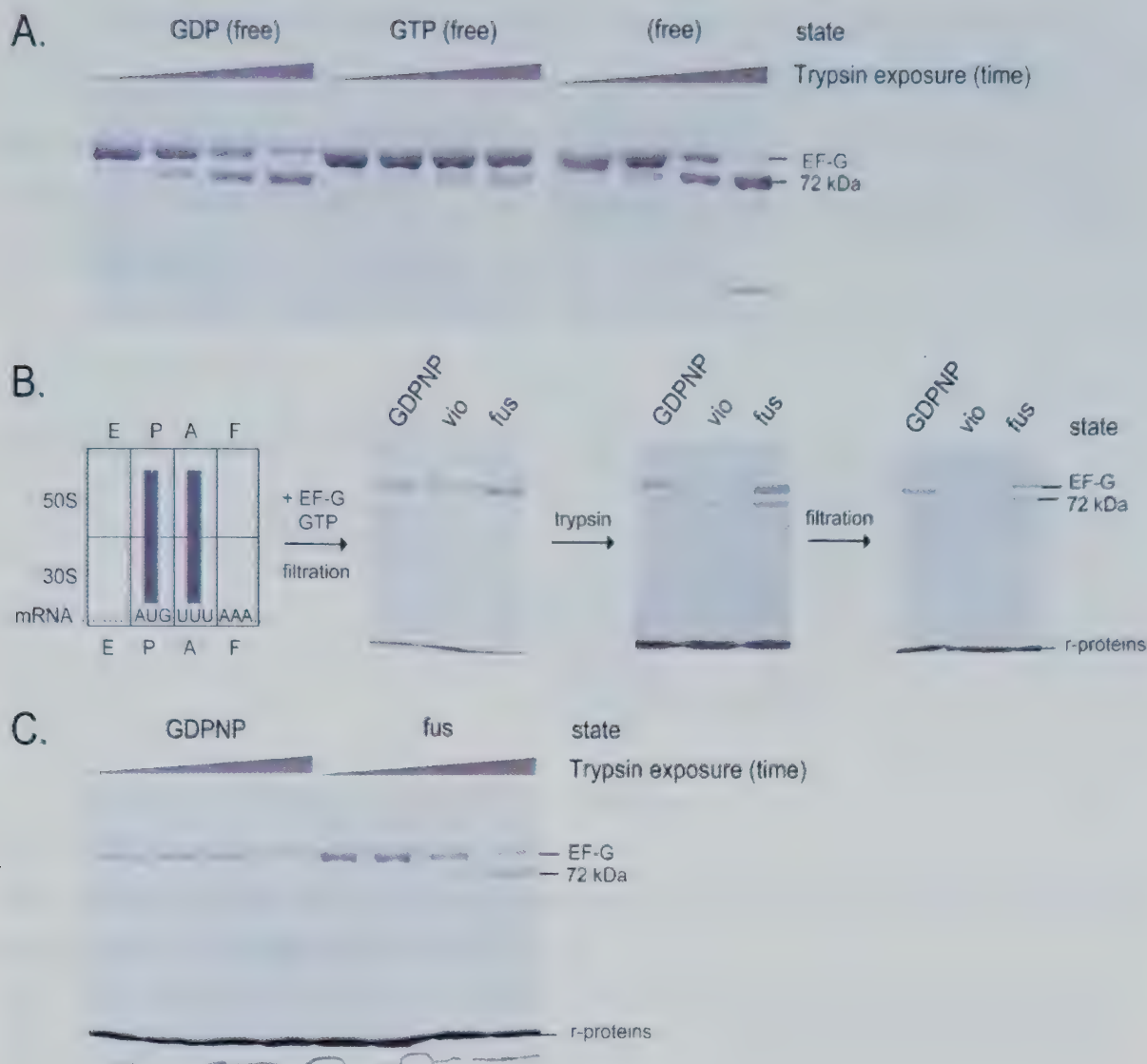


Fig. III.3. Trypsin probing of the six EF-G states

A. Free states: complexes of 2 μM EF-G and 0.5 mM each of GDP or GTP were probed with 4.5 $\mu\text{g}/\mu\text{l}$ trypsin. Aliquots (removed at 1, 4, 16, and 64 minutes) of each reaction were denatured and analyzed by 10-12 % SDS-PAGE.

B., C. Ribosome-bound states. (B) pre-translocational ribosomal complexes were formed with 2 μM ribosomes, 2.4 μM mRNA, 2.4 μM tRNA^{Met} (P site) and 2.4 μM tRNA^{Phe} (A site). The EF-G states were formed with 1.7 μM EF-G and 0.5 mM of each GDPNP, GTP, GDP, viomycin and fusidic acid, as described in Fig. III.2., C. and D. (also see III.6.5.). All complexes were then filtered to discard free EF-G, followed by 10 minutes digestion with 4.5 $\mu\text{g}/\mu\text{l}$ trypsin. The digested complexes were then re-purified by filtration to discard dissociated/free EF-G, denatured and analyzed as in panel A. (C) GDPNP and fus states were formed and filtered as described in panel B., followed by trypsin digestion and analysis as described in panel A.

Data in this figure were obtained by Roxana Nechifor and modified from [23].

In order to identify the trypsin cleavage site, we determined the N-terminus sequence of the 72 kDa fragment by Edman degradation, and found that the trypsin cleaved peptide bond is located at the tip of the switch I region, between residues 59 and 60 (*E. coli* numbering is used throughout this chapter) (Fig. III.4., A.).

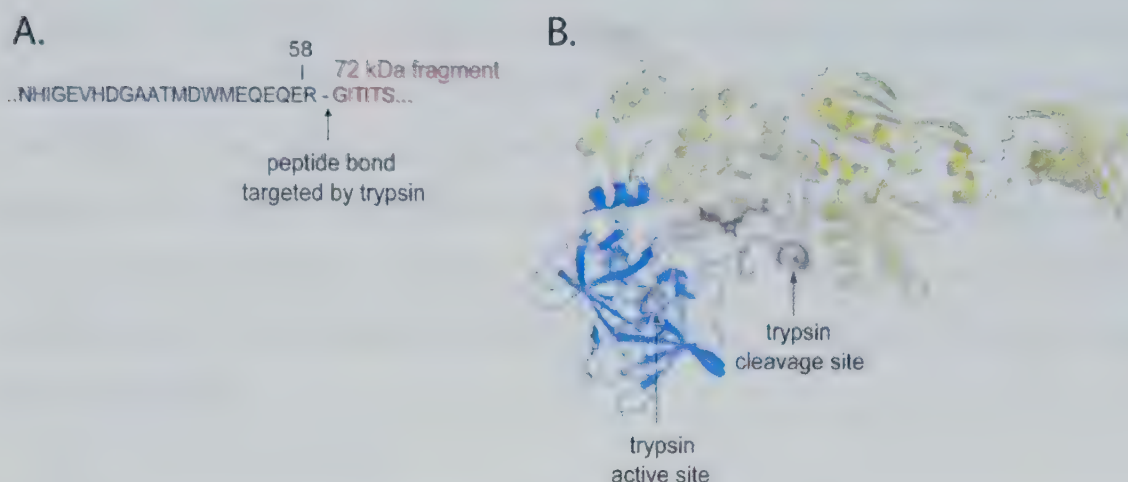


Fig. III.4. Identification of the tryptic cleavage site and modeling its spatial interactions with the enzyme's active site

A. The *E. coli* primary sequence of the switch I region of EF-G is shown from N to C terminus. The N terminus sequence of the 72 kDa fragment (red) is marked.

B. Using Pymol modeling, trypsin (blue) was docked onto the *T. thermophilus* EF-G-2·GDPNP structure (yellow) [19] (PDB file 1WDT). For simplicity, the ribosome is invisible. The trypsin active site and the cleaved peptide bond in switch I (tan) are highlighted in red and marked by arrows.

The Edman degradation was done by Roxana Nechifor (results shown in panel A.) and the Pymol modeling was done by Kevin Wilson (panel B.). Both panels are reproduced with modifications from [23].

The switch I region is highly conserved among translational GTPases, and an identical cleavage site had been previously observed after the corresponding arginine residues in eEF-1 α [34] and EF-Tu [35], as well as after the corresponding eEF-2 arginine residue [36]. The latter study however reported switch I to be similarly susceptible to trypsin degradation in the GDP and GTP free states, and only protected from trypsin attack while bound to the ribosome with a GTP/GDP analog, called FSBG (5'-p-fluorosulfonylbenzoylguanosine).

We attempted to model trypsin interaction with the switch I region of ribosome bound EF-G by using the only available structure of EF-G on the ribosome containing an ordered switch I region: [19]. By docking the trypsin molecule at the entrance to the ribosomal inter-subunit cavity, below the bound EF-G (Fig. III.4., B.), it becomes obvious why the GDPNP state showed little to no cleavage of the switch I loop (Fig. III.3., B. and C.): the cleaved peptide bond between R59 and G60 in switch I is spatially inaccessible to the active site of the trypsin molecule. However, if the switch I loop were to change its conformation and adopt a more “open” or “outward” spatial orientation, the serine residue at the active site of trypsin could plausibly access the determined EF-G tryptic cleavage site. Another plausible scenario is that switch I remains in its tucked in conformation, but becomes passively accessible to trypsin during ribosome ratcheting [37].

III.2.3. Ribosomal ratcheting does not affect switch I accessibility to trypsin

The unratcheted ribosome has a peptidyl-tRNA in a P/P site and an aa-tRNA in the A/A site. Ribosomal subunits ratchet upon peptide bond formation and 50S translocation [9, 38] bringing the new peptidyl-tRNA into the A/P position and the deacylated, formerly P/P tRNA, into the P/E state, movement known as “hybrid state formation” [39]. Ribosomal ratcheting can be stabilized by EF-G binding in the presence of GDPNP or fusidic acid [9, 32] or in the absence of EF-G by the antibiotic viomycin [22].

In order to distinguish between the two above mentioned scenarios, we assembled ribosomal complexes with EF-G in the ratcheted or unratcheted states [40] and probed the fus state for trypsin attack. As diagrammed in Fig. III.5., we started with a vacant ribosome (“v”) and bound *N*-acetyl-Phe-tRNA^{Phe} (*N*-Ac-Phe-tRNA^{Phe}) to the P/P site, obtaining the unratcheted ribosomal state (“u”). The addition of the antibiotic puromycin leads to a non-hydrolysable amide bond formation between *N*-Ac-Phe-tRNA^{Phe} and puromycin. The *N*-Ac-Phe-puromycin product is subsequently released from the ribosome, and as the deacylated tRNA^{Phe} moves in the P/E position, hybrid state formation is achieved (“r”).

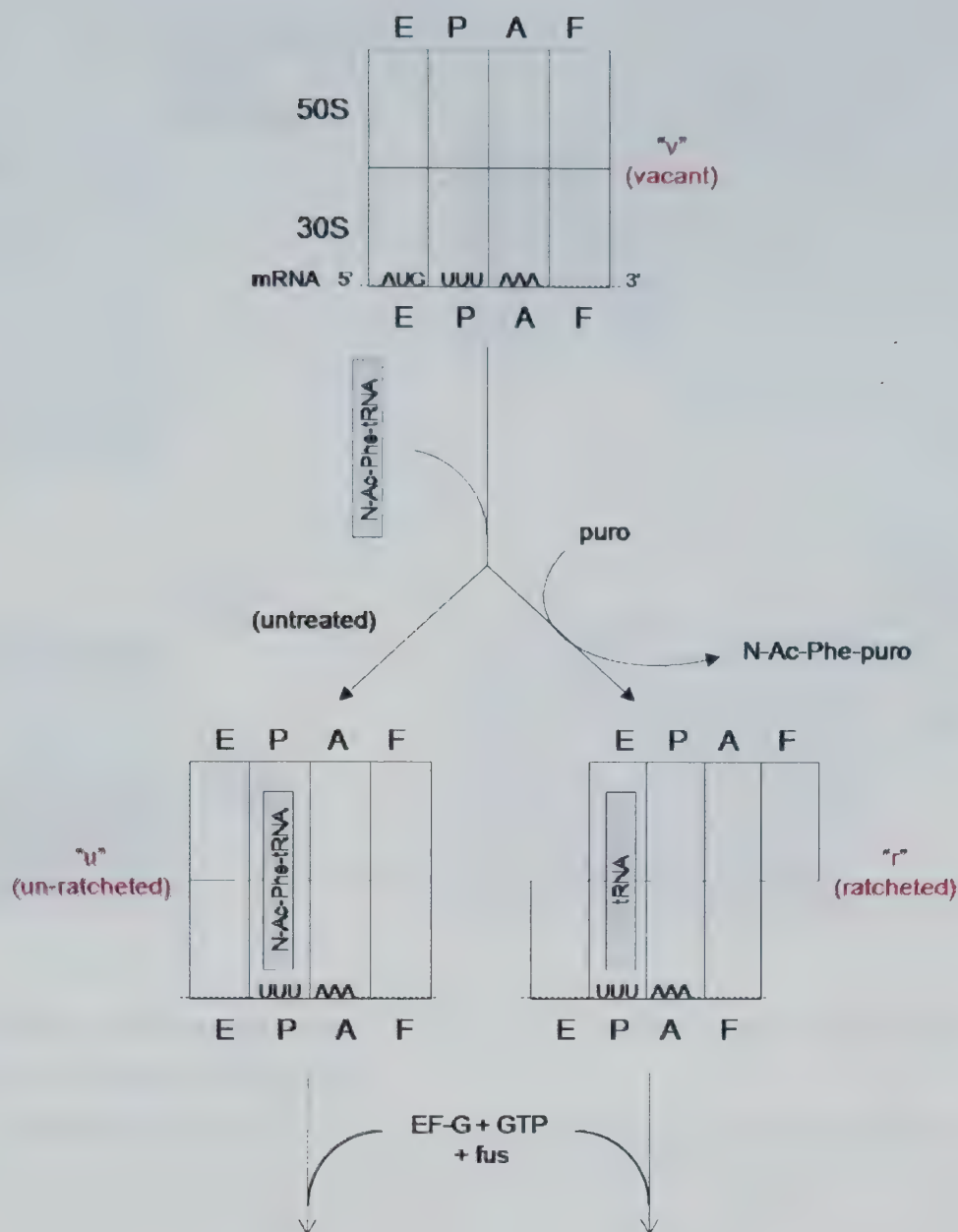


Fig. III.5. Trapping the ribosome in v, u or r states

The ribosomal tRNA binding sites are as described in Fig. III.1. The vacant ("v"), unratcheted ("u") and ratcheted ("r") states are marked in red.

We first evaluated whether $N\text{-Ac-Phe-tRNA}^{\text{Phe}}$ correctly binds to the P site of the pre-translocational ribosome. Toeprinting experiments showed $N\text{-Ac-Phe-tRNA}^{\text{Phe}}$ efficiently (comparable with tRNA^{Met} and tRNA^{Phe}) and correctly (to the UUU codon situated in the P site) bound to the ribosome (Fig. III.6., A.). We then examined EF-G binding to the u and r complexes, by mapping EF-G dependent protection of nucleotide A2660 [41] in the 23S rRNA of the 50S ribosomal subunit from DMS modification (Fig. III.6., B.).

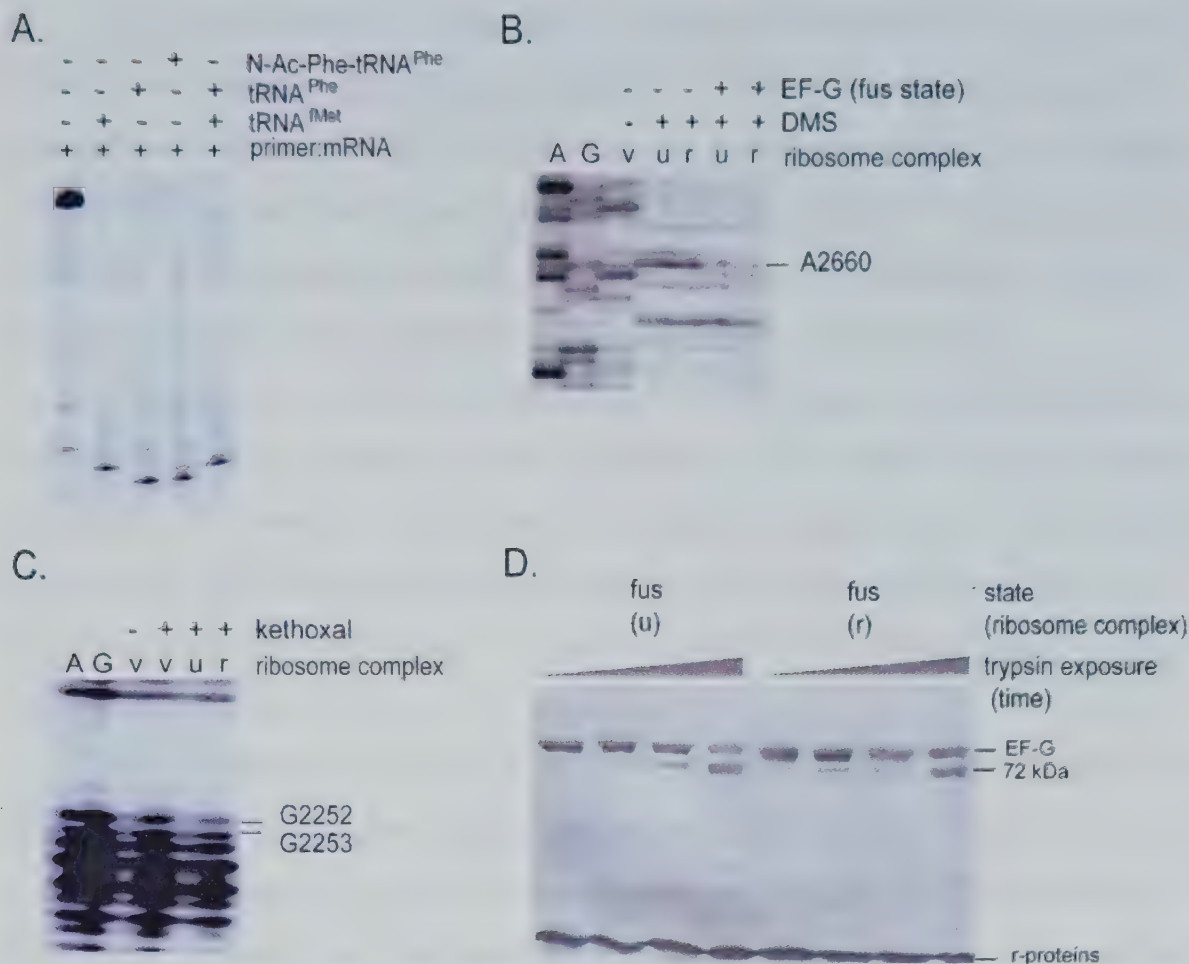


Fig. III.6. Ribosomal u and r complexes exhibit similar degrees of trypsin exposure of the switch I loop

A. Toeprinting experiment was conducted under the same conditions as in Fig. III. 2., C., except in lane 4, 1.2 μM $N\text{-Ac-Phe-tRNA}^{\text{Phe}}$ was added to a v complex.

B., C. Chemical probing of rRNA in the u and r complexes. 2 μM ribosomes and 2.4 μM mRNA (v) bound 2.4 μM $N\text{-Ac-Phe-tRNA}^{\text{Phe}}$ to the P site (u) [39]. 1 mM puromycin was incubated with the u complex for 30 minutes at 20° C (r).

V, u and r complexes were probed with DMS (B.) or kethoxal (C.) in presence or absence of 1.7 μM EF-G. EF-G binding was assessed by protection from DMS rRNA modification at A2660 [41] and $N\text{-Ac-Phe-tRNA}^{\text{Phe}}$ presence in the 50S P site was assessed by protection from kethoxal modification at nucleotides G2252 and G2253 in 23S rRNA [42]. The chemical modification of the 23S rRNA was monitored by primer extension [43]

D. Trypsin probing of the u and r complexes in the fus state: u and r complexes were assembled as in panels B. and C., followed by trypsin probing as described in Fig. III.3., C.

Data in panel D. were obtained by Roxana Nechifor. Panels B., C., and D. are reproduced with modifications from [23].

We then investigated the u and r ribosomal complexes for appropriate hybrid state formation. By monitoring 50S 23S rRNA for P site protection at nucleotides G2252 and G2253 from kethoxal modification [42], we confirmed that the u ribosome states indeed contained *N*-Ac-Phe-tRNA^{Phe} in the 50S P site, while the r ribosome underwent 50S translocation, corresponding to lack of protection at 50S P site nucleotides G2252 and G2253 (Fig. III.6., C.).

Based on the information provided to us by these essential controls, we then proceeded to compare the susceptibility of the switch I loop to tryptic cleavage in the presence or absence of ribosomal ratcheting. By reproducing the previous fus state time-based trypsin cleavage experiment conditions (Fig. III.3., C.), we found no difference in trypsin susceptibility between the u and r complexes (Fig. III.6., D.).

We therefore concluded that ribosomal ratcheting is most likely not responsible for the higher trypsin accessibility of the GTP states of EF-G compared with the GDP states. We therefore investigated the possibility that GTP hydrolysis induces a yet uncharacterized conformational change in the position and/or orientation of the switch I loop, thus rendering it more accessible to trypsin cleavage.

III.2.4. Upon GTP hydrolysis and 30S translocation, the switch I loop flips out from the ribosomal cavity

In order to monitor a hypothetical conformational change of switch I with respect to the ribosome, we chose a hydroxyl radical generating probe, **Fe(II)-bromo-acetamidobenzyl-EDTA (FeBABE)** (Fig. III.7.) which covalently attaches to thiol groups (i.e. cysteine residues). In the presence of ascorbic acid and low concentration (0.1 % v/v) of hydrogen peroxide, the FeBABE probe generates diffusible hydroxyl radicals which can cause backbone cleavages at nearby rRNA sites (Fig. III.7.). The hydroxyl radicals are short lived (due to the reducing ability of the ascorbic acid) and their activity is limited to less than 25 Å distance, although they may be able to travel in solution (in a spatially unrestricted environment) to up to 40 Å away from the FeBABE probe [44].

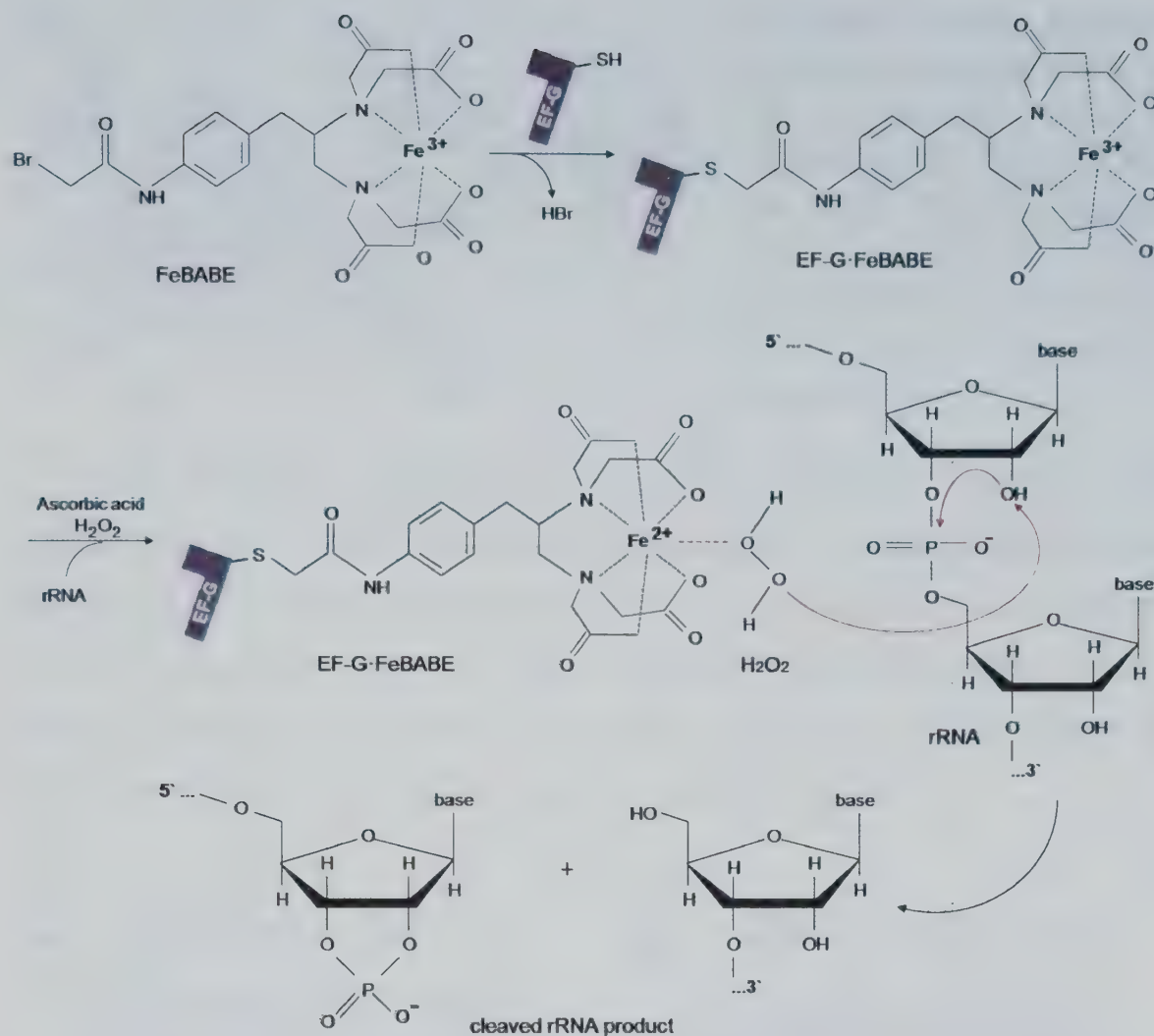


Fig. III.7. The chemistry behind FeBABA hydroxyl radical probing of rRNA
 The schematic EF-G representation is as in Fig. III.1. The hydrogen peroxide coordinates (red) with the Fe^{3+} which subsequently becomes Fe^{2+} . The mechanism of hydrophilic attack of the rRNA backbone is represented with red arrows.

We engineered several single cysteine mutants (Fig. III.8.) in a cysteine free version of *E. coli* EF-G [45]. Four of these residues, 37, 58, 65 and 196, were labeled with FeBABA to monitor the movement of switch I by hydroxyl radical probing. Residue 58 is located adjacent to the trypsin cleavage site, at the tip of the switch I loop, 35 and 67 flank the switch I region, while 196 is an external residue, used as a technical control. This residue was chosen because the rRNA 196C-FeBABA fus state cleavages have already been characterized [45] and because it is situated in a region of EF-G which is not expected to significantly change position with respect to the ribosome during the EF-G cycle.

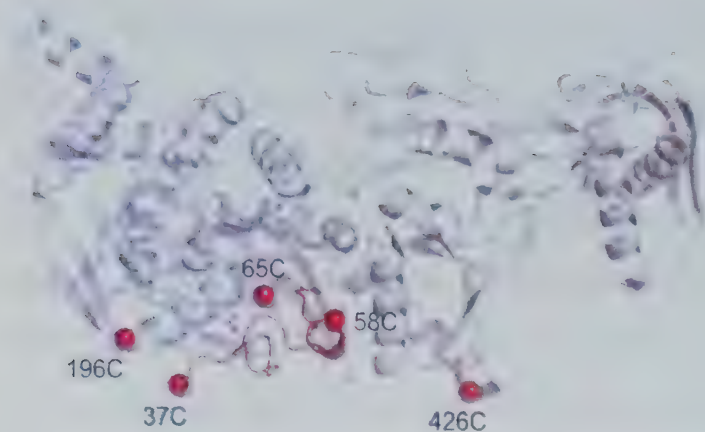


Fig. III.8. Location of single cysteine mutants in EF-G

EF-G (PDB file 1WDT) is shown in white. Switch I and the single cysteine mutants used in this study are colored red. Spheres are centered on the Ca atoms of residues of interest.

We started by labeling our four single cysteine EF-G mutants with FeBABE [45]. Our FeBABE labeling procedure involves a post-labeling filtration step (see III.6.6.), which removes the un-reacted FeBABE probe, thus reducing the risk of false positive results. We then evaluated the FeBABE labeled proteins' ability to hydrolyze GTP under single turnover conditions and translocate the mRNA and tRNAs in the GDPNP, vio and fus states (Fig. III.9.).

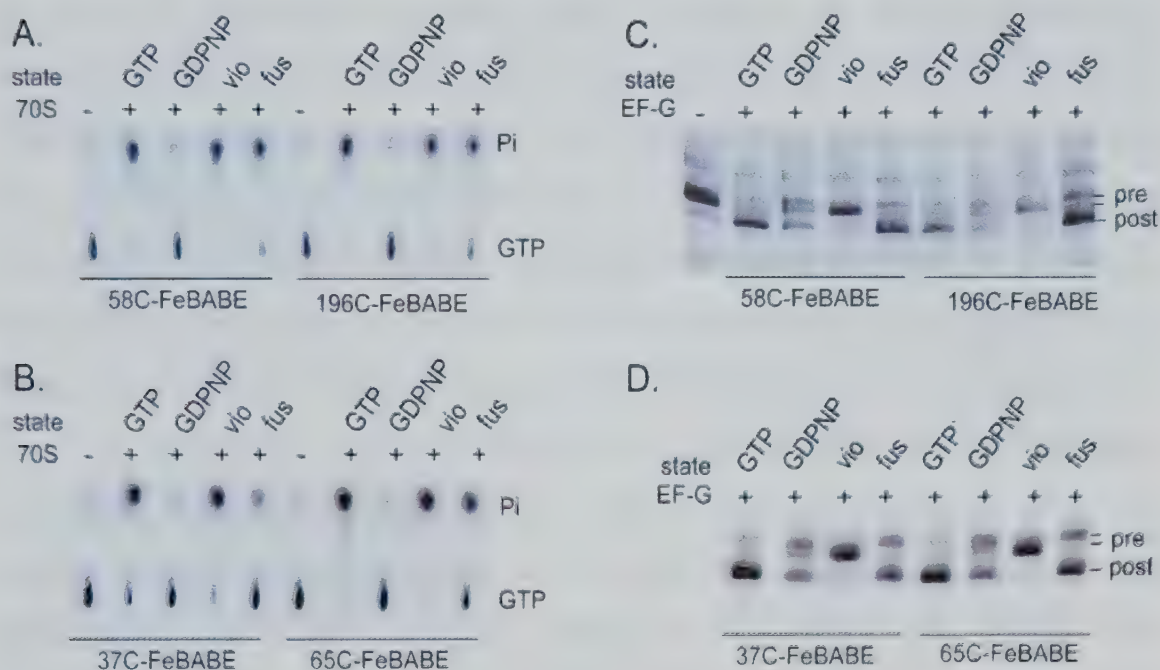


Fig. III.9. The FeBABE proteins are functional

A., B. GTP hydrolysis. Experiments were conducted using FeBABE labeled proteins under single turnover conditions as described in Fig. III.2., A.

C., D. Translocation. All lanes in panels C. and D. contain pre-translocational ribosomal complexes. Toeprinting experiments were conducted using FeBABE labeled proteins as described in Fig. III.2., C.

Panels A. and C. are reproduced with modifications from [23].

We found that all FeBABE labeled proteins behaved as wild type EF-G (Fig. III.2.) in terms of GTP hydrolysis. 58C-FeBABE and 196C-FeBABE translocated in all three ribosome bound states similar to the wild type protein (Fig. III.9., A. and B.), however 37C-FeBABE and 65C-FeBABE showed slight impairment in translocation in the fus state, by comparison with wild type and the other two FeBABE labeled proteins (Fig. III.9., D.).

We then bound the FeBABE labeled proteins to the ribosome in all three states and generated hydroxyl radicals by addition of hydrogen peroxide. As a control, we bound FeBABE-treated cysteine free EF-G to the ribosome in the fus state and treated the sample with hydrogen peroxide in parallel with the other EF-G single cysteine mutants.

The rRNA was then precipitated and extracted [45], and both 16S and 23S rRNAs were scanned for FeBABE dependent cleavages by primer extension [26, 43] (Fig. III.10). As predicted, our negative control of cysteine free EF-G mutant showed no FeBABE dependent rRNA cleavage. In the fus state, the 196C-FeBABE control cleaved the exact same nucleotides in helix 95 (SRL) as previously reported by the first study to map the EF-G position on the ribosome in the post-translocational (fus) state [45] (Fig. III.10., A.). Also, the cleavage pattern given by 196C-FeBABE was invariable throughout the three ribosome bound states, suggesting that residue 196 is not subjected to a great deal of motion during the progression of the EF-G cycle from GTP hydrolysis to translocation.

In the scanned 16S rRNA regions, the GDPNP state shows no detectable cleavages, while the vio state showed similar cleavages as the fus state, only weaker, which can be attributed to the decreased stability of the vio state. Interestingly, all switch I mutants cleaved the 16S rRNA only in their post-translocational (fus) state. 58C-FeBABE and 65C-FeBABE gave identical RNA cleavages. 37C-FeBABE gave much weaker, but noticeable, cleavages in the same regions as its fellow switch I mutants (Fig. III.10., B.-E.). 196C-FeBABE did not cleave any of the 16S rRNA nucleotides cleaved by the switch I mutants, except for some very weak cleavages close to nucleotide 334 (Fig. III.10., F.).

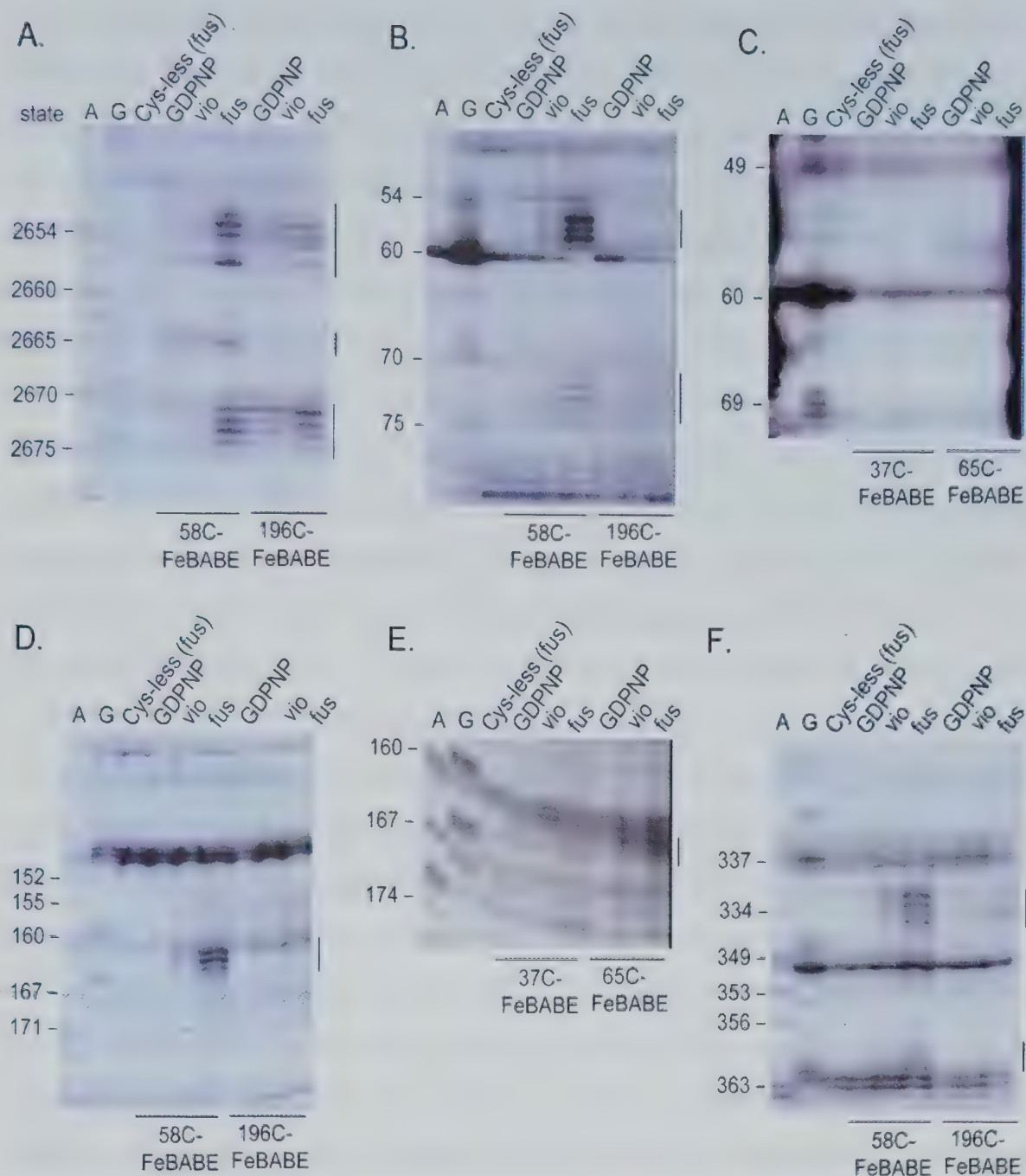


Fig. III.10. Primer extension analysis of hydroxyl radical probing

2 μM FeBABE labeled mutants were bound to pre-formed pre-translocational ribosome complexes (0.5 μM ribosomes with 1 μM each of mRNA and tRNAs) in presence of 0.5 mM each of GDPNP, GTP, viomycin or fusidic acid, as indicated. Hydroxyl radical probing was allowed to proceed for 10 minutes on ice and then quenched with 166 mM thiourea. The rRNA was purified by extensive extraction procedures and analyzed by primer extension [26, 43]. The first two lanes (A, G) are rRNA sequencing reactions and the corresponding *E. coli* rRNA nucleotides are numbered on the left side of the gels. Panel A scans the 23S rRNA and panels B., C., D., E. and F. examine the 16S rRNA.

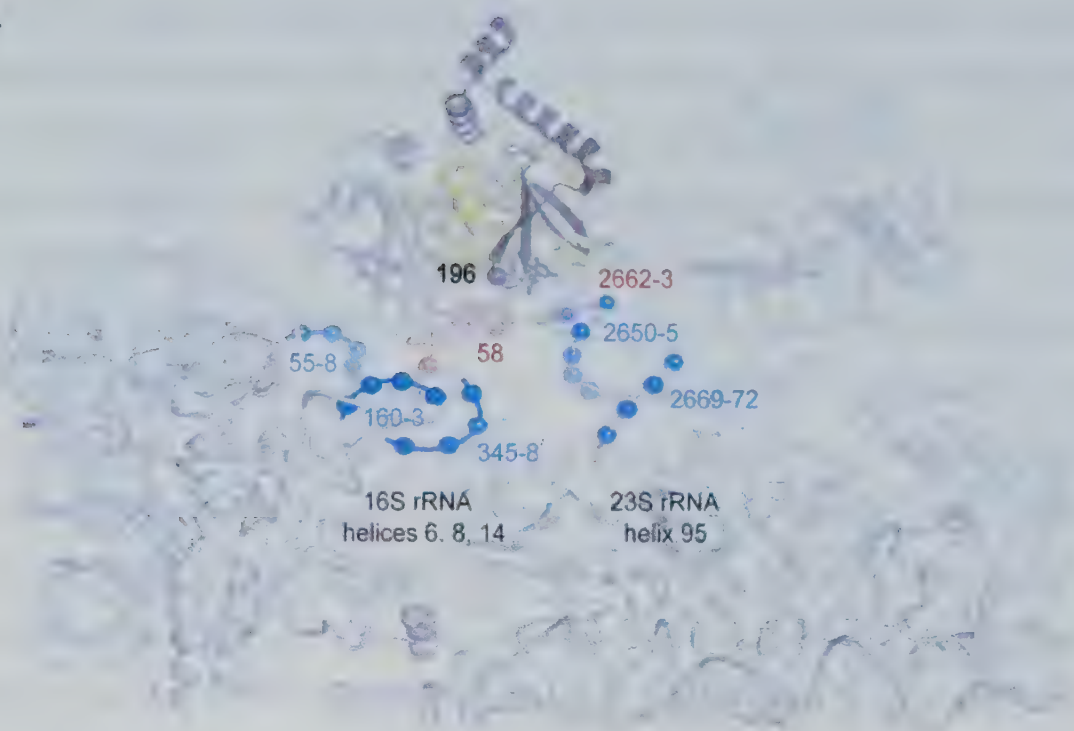
Panels A., B., D. and F. are reproduced with modifications from [23].

In the 23S rRNA (Fig. III.10., A.), in the fus state, 58C-FeBABE targeted nucleotides close to the base of helix 95, which was a similar cleavage pattern as the one previously reported for 196C-FeBABE in the fus state [45]. However, two other nucleotides (2662, 2663) at the tip of helix 95 were also distinctly cleaved by 58C-FeBABE. In the vio state, the same two nucleotides were cleaved, together with weakly cleaved nucleotides in the stem of helix 95, while in the GDPNP state, 2662 and 2663 were the only cleaved nucleotides.

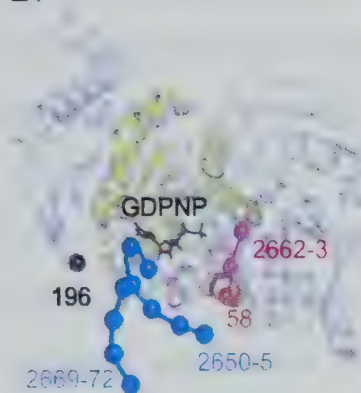
Since residue 58C is closest to the tryptic cleavage and therefore the adjacent switch I portion most likely withstands the most extensive conformational changes, we mapped the nucleotides cleaved by the 58C-FeBABE protein onto the crystal structure of *T. thermophilus* EF-G-2·GDPNP (PDB file 1WDT) superimposed on a cryo-EM map of ribosome bound EF-G·GDPNP (PDB file 2OM7) [19] (Fig. III.11., A. and B.). By examining the RNA cleavages given by 58C-FeBABE and 196C-FeBABE in the above EF-G-ribosome complex, only the 196C-FeBABE targeted cleavages sterically match the position of the EF-G residue with respect to the rRNA. Indeed, the hydroxyl radicals generated by 196C-FeBABE would have a clear line of attack towards the observed 23S cleavages, as residue 196 is located outside of the ribosomal cavity.

In the GDPNP state, the 23S rRNA cleavages given by 58C-FeBABE are in agreement with the structure of EF-G-2·GDPNP on the ribosome [19], as nucleotides 2662 and 2663 are situated at only 16 and 9 Å away from 58C, respectively. In the fus state however, the 23S rRNA cleavages tell a different story. Firstly, residue 58C (and therefore switch I) is buried inside the ribosomal cavity, and helix 95 would sterically interfere with the diffusion path of the hydroxyl radicals towards the 23S residues cleaved (2650-2655 and 2669-2672). Secondly, these residues are situated approximately 35 Å away from 58C, which is verging on the distance limitations of our hydroxyl radical probing technique, and yet the 58C-FeBABE cleavages in this region are even stronger than the ones given by 196C-FeBABE. Thirdly, the cleavages in 16S rRNA are located even further away from the position of residue 58 as shown by the EF-G-2·GDPNP crystal structure [19].

A.



B.



C.



Fig. III.11. Theoretical mapping of switch I conformational changes

A. 58C-FeBABE cleavages: EF-G-2 (PDB file 1WDT) [19] is dark gray (except for the G domain - yellow and the switch I region - red) and the surrounding rRNA (PDB file 2OM7) [19], in light gray. Residues 58 and 196 are shown in red and black respectively, with spheres centered on the C_{α} atoms. The cleaved rRNA nucleotides are shown in magenta (GDPNP) and blue (GDPNP and fus), with spheres centered on the P_{α} atoms.

B. Switch I (GDPNP). The same data and color codes as in panel A are used, except the body of EF-G-2 is colored in light gray, the ribosome is invisible, except for the cleaved nucleotides in helix 95.

C. Switch I (fus). EF-G(G16V)-GDP (PDB file 2BM1) [13] was superimposed on the ribosome structure used in panels A. and B. The color code and limited ribosomal RNA display is as in panel B.

The Pymol modeling was done by Kevin Wilson and modified from [23].

Our hydroxyl radical probing data can be reconciled if residue 58 were to withstand a conformational change between the GDPNP and the fus state. Before GTP hydrolysis, switch I could be tucked inside the ribosomal cavity, which would explain the limited, two nucleotide, cleavages we observed in the GDPNP state for 58C-FeBABE (Fig. III.10., A.) and the limited access of trypsin to switch I in the GDPNP state (Fig. III.3., B. and C.). However, if following 30S translocation and GTP hydrolysis, residue 58 moves out of the ribosomal cavity, beyond helix 95, then both the fus state cleavages given by 58C-FeBABE can be explained in terms of diffusion of hydroxyl radicals, both sterically and distance-wise (Fig. III.11., A.). Also, such exposure of switch I in the fus state would explain the higher susceptibility to trypsin cleavage of the switch I loop compared with the protected, GDPNP state (Fig. III.10., C.). Moreover, as switch I flips out of the ribosomal cavity, it loses anchorage with the rest of EF-G and oscillates randomly in solution between open and closed states, which explains the lack of crystal ordering observed by numerous crystallographic studies [11-15, 20].

Curiously, the crystal structure of a fusidic acid sensitive mutant of EF-G, G16V·GDP [13], which has an overall more “closed” conformation in solution compared with wild type EF-G and has been suggested to mimic the post-translocational (fusidic acid accessible) structure of EF-G on the ribosome [15], has a partially ordered switch I, which is projected away from the G domain (Fig. III.11., C.). Also, the fus state of EF-G shows a partially ordered switch I which seems to be exposed in solution [20], which is consistent with our findings.

III.2.5. EF-G and GDP are released faster from the ribosome in the states corresponding with a flipped-out conformation of switch I

Previous functional studies of EF-G kinetics on the ribosome report strong increases in the binding equilibrium constants for GDPNP [46], GTP and GDP [47] in the presence of the ribosome. Also, the dissociation rate constant for GDPNP has been shown to decrease by approximately 10⁴ fold compared with GTP and GDP [46]. In the context of structural studies showing switch I to be tucked in close to the α phosphate of GDPNP on the ribosome bound EF-G-2 [19]

and extended away from EF-G in the fus state [20], as well as in light of our own findings, we wondered whether switch I conformational changes are related to nucleotide release, and in turn, to EF-G release and recycling.

To address these hypotheses, we attached two fluorescent probes to EF-G and either GTP, GDP or GDPNP, and measured their respective release rates from ribosome complexes, formed in such a way as to reflect the GDPNP, vio and fus states. Oregon green (OG) was attached to EF-G(426C) and EF-G release from the ribosome was monitored with respect to the OG probe fluorescence increase. We chose this particular single cysteine mutant since it is situated away from the GTPase center of EF-G (Fig. III.8.) and therefore accurately reflects EF-G release from the ribosome rather than conformational changes within the G domain triggered by GTP hydrolysis. We formed EF-G(426C-OG)-ribosome complexes in presence of GDPNP (for the GDPNP state) or viomycin/fusidic acid and GDP (for the vio/fus states respectively), followed by rapid mixing with excess wild type EF-G. Thus the dissociation rate of EF-G(426C-OG) from the ribosome was measured by quantifying the time dependent OG fluorescence increase. To measure the kinetics of nucleotide dissociation from the ribosome, we quantified the fluorescence decrease of mant-GDP. EF-G-ribosome complexes were formed in presence of mant-GDPNP (for the GDPNP state) or viomycin/fusidic acid and mant-GDP (for the vio/fus states respectively). The complexes were rapidly mixed with excess unlabeled nucleotide (GDPNP or GDP, respectively), and the mant-GDPNP/GDP dissociation rate was monitored by quantification of the fluorescence decrease over time.

We used vacant ribosomes for these assays because, firstly, each stopped flow shot requires reaction volumes of approximately 400 μ l, therefore these experiments consume a significant amount of materials. Secondly, previous studies have shown that the presence of tRNA and mRNA in the ribosomal complex does not affect the GTPase activity of EF-G [27]. Also, due to interference between the fluorescence spectra of our two fluorescent molecules (mant and Oregon Green), we could not simultaneously monitor the release of both nucleotide and EF-G.

We started by examining the release of EF-G and nucleotide from the GDPNP and GDP complexes (Fig. III.12.). We found that EF-G (GDPNP state) and mant-GDPNP were released about 860 and 7500 fold slower, respectively, into solution compared with EF-G (GDP state) and mant-GDP (Fig. III.12., Table III.1.). The 5.8 fold difference between the k_d values obtained for nucleotide and EF-G release in the GDPNP state is in agreement with the previously reported three fold difference [46], which was attributed to the EF-G binding stabilizing effect of the mant group.

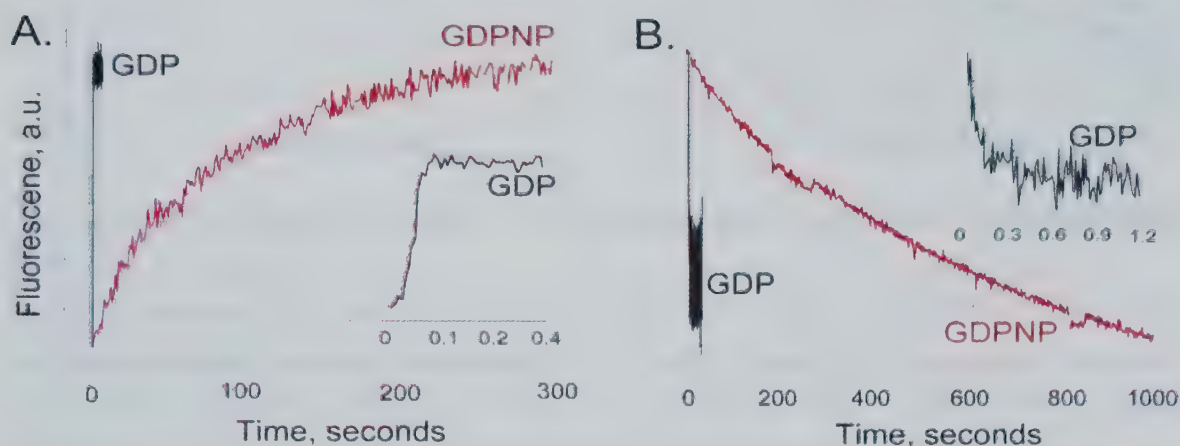


Fig. III.12. Probing GDPNP and GDP states release kinetics of EF-G and nucleotide from the ribosome

A. EF-G release: Pre-incubated 2 μ M EF-G(426C-OG), 3 μ M 70S and 0.2 mM of either GDPNP (red) or GDP (black) were rapidly mixed with an equal volume of 20 μ M wild type EF-G. The inserted lower graph shows a zoomed in version of EF-G(426C-OG) release from the ribosome in the GDP state.

B. Nucleotide release: Pre-incubated 2 μ M wild type EF-G, 3 μ M 70S and 2 μ M mant-GDPNP (red) or mant-GDP (black) were rapidly mixed with an equal volume of 2.4 mM GDPNP (red) or GDP (black). The inserted upper graph shows a zoomed in version of mant-GDP release from EF-G and the ribosome in the GDP state.

We then investigated the kinetics of EF-G and nucleotide release from the ribosome in the fus and vio states (Fig. III.13.). EF-G-ribosome-vio/fus complexes were formed as described above and rapidly mixed with the excess corresponding antibiotic. Both the vio and fus states released mant-GDP 500 and 22 fold faster, respectively, than the GDPNP state complex released mant-GDPNP (Table III.1.).

Also, both *vio* and *fus* states released mant-GDP 15 and 750 fold slower, respectively, than the GDP state. EF-G was also released slower than the GDP state (14 and 186 fold, respectively).

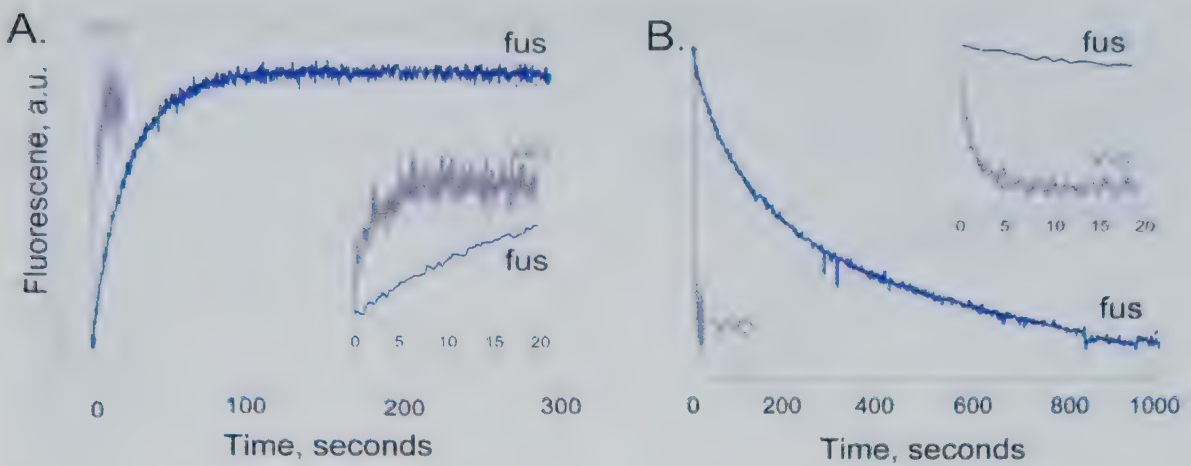


Fig. III.13. Probing *vio* and *fus* states release kinetics of EF-G and nucleotide from the ribosome

A. EF-G release Pre-incubated 2 μM EF-G(426C-OG), 3 μM 70S, 0.2 mM GDP and 0.1 mM *vio* (gray) or *fus* (blue) were rapidly mixed with an equal volume of 20 μM wild type EF-G. The inserted lower graph shows a zoomed in version of EF-G(426C-OG) release from the ribosome in the *vio* state.

B. Nucleotide release Pre-incubated 2 μM wild type EF-G, 3 μM 70S, 2 μM mant-GDP and 0.1 mM *vio* (gray) or *fus* (blue) were rapidly mixed with an equal volume of 2.4 mM GDP. The inserted upper graph shows a zoomed in version of mant-GDP release from EF-G and the ribosome in the *vio* state.

In order to investigate the *vio* effects further, we first evaluated whether the above fast release of EF-G and nucleotide from the *vio* complex is biased by EF-G being able to exchange mant-GDP with GDP while still bound to the ribosome. We formed complexes with ribosomes, viomycin, OG labeled EF-G and mant-GDP and rapidly mixed them with either excess EF-G or excess EF-G and GDP. If in the *vio* state EF-G would be able to exchange GDP while still on the ribosome, the second experiment would exhibit a slower release of EF-G(426C-OG) from the ribosome, as the excess GDP would stabilize the protein while replacing the mant-GDP on EF-G.

As shown in Fig. III.14. we found this hypothesis to be incorrect, as both EF-G release experiments revealed very similar k_d values (3.26 vs 3.21 s⁻¹). This result indicates that the vio complex is simply less stable in terms of EF-G binding to the vio-trapped ratcheted ribosome, therefore the release of both EF-G and nucleotide is accelerated compared with the fus complex.

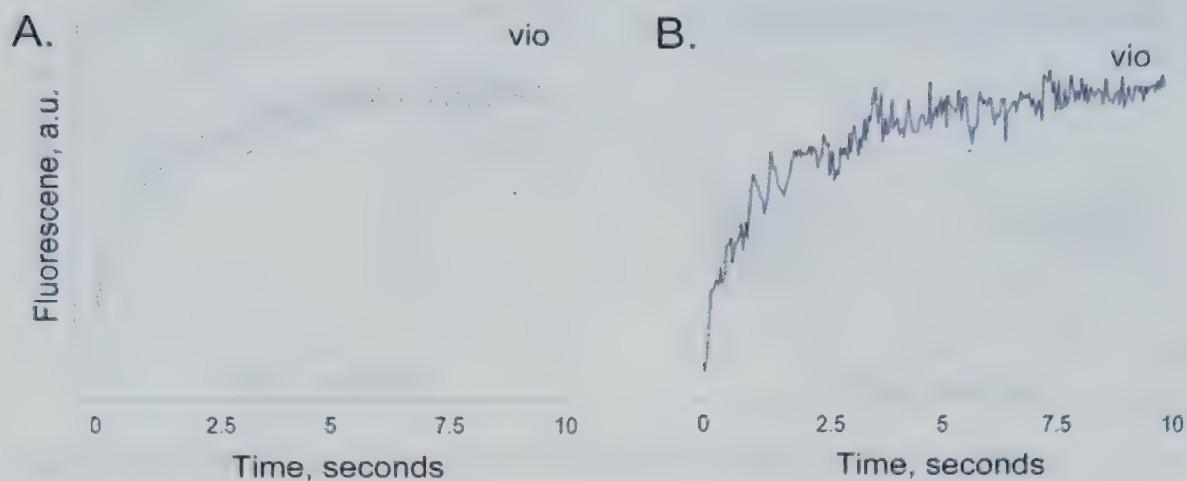


Fig. III.14. Probing the vio state for EF-G release kinetics

Pre-incubated 2 μ M EF-G(426C-OG), 3 μ M 70S, 10 μ M GDP and 0.1 mM vio were rapidly mixed with either 10 μ M wild type EF-G (light gray) (A.) or 10 μ M wild type EF-G and 1 mM GDP (dark gray) (B.).

In order to further investigate the fus state, EF-G·GDP·ribosome complexes were chased with either GDP alone or with a combination of GDP and fusidic acid (Fig. III.15.). Although the fusidic acid binding site has already been identified by crystallography [20], the conformational changes of switch I triggered by GTP hydrolysis may be part of EF-G's fusidic acid sensitivity mechanisms, in the sense that switch I movement towards the outside of the ribosomal cavity may open the entry pathway for fusidic acid binding. This hypothesis is supported by crystallographic studies of apo EF-G fusidic acid mutants, which had extensive rearrangements of their switch regions within the G domain and had an overall more closed conformation compared with wild type EF-G [13-15]. If so, the mant-GDP release should be significantly slowed by fusidic acid addition.

Indeed, fusidic acid immediately stabilized both EF-G and the nucleotide on the ribosome even when EF-G was not pre-bound to the ribosome. Also, these results indicate that fusidic acid traps EF-G·GDP on the ribosome soon after the first round of GTP hydrolysis.

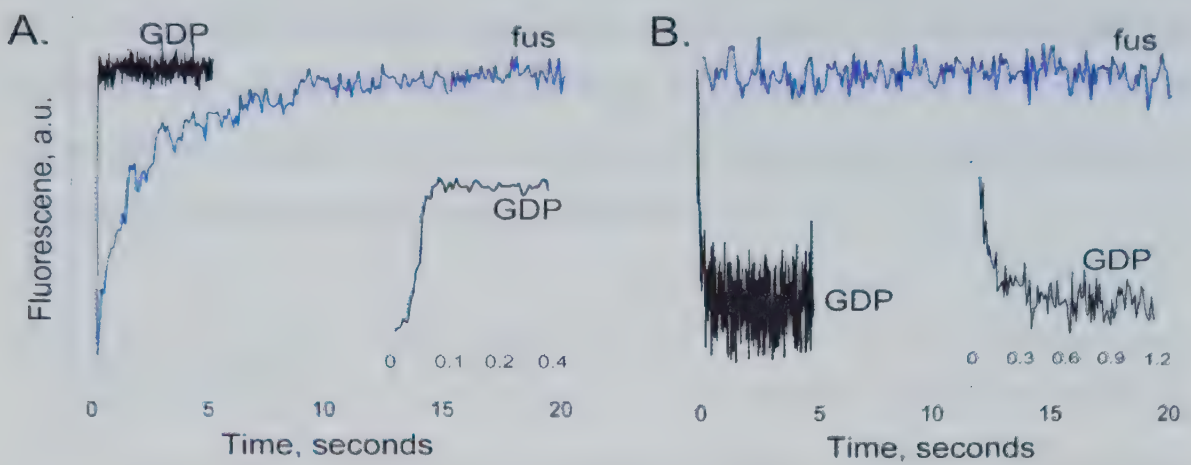


Fig. III.15. Fusidic acid readily stabilizes EF-G·GDP on the ribosome

A. EF-G release Pre-incubated 2 μM EF-G(426C-OG), 3 μM 70S and 0.2 mM GDP were rapidly mixed with an equal volume of either 20 μM wild type EF-G (black) or 20 μM wild type EF-G and 0.1 mM fus (blue). The inserted lower graph shows a zoomed in version of EF-G(426C-OG) release from the ribosome in the GDP state.

B. Nucleotide release Pre-incubated 2 μM wild type EF-G, 3 μM 70S and 2 μM mant-GDP were rapidly mixed with an equal volume of 2.4 mM GDP (black) or 2.4 mM GDP and 0.1 mM fus (blue). The inserted lower graph shows a zoomed in version of mant-GDP release from EF-G and the ribosome in the GDP state.

Table III.1. EF-G and nucleotide release from the ribosome

Ribosome bound EF-G state	Dissociation constant, k_d , s^{-1} *	
	EF-G release	Nucleotide release
GDPNP	0.0093 ± 0.0005	0.0016 ± 0.0001
GDP	8 ± 2	12 ± 2
Vio	0.56 ± 0.15	0.8 ± 0.09
Fus	0.043 ± 0.001	0.016 ± 0.005
GDP stabilized by fus	0.42 ± 0.05	0.019 ± 0.002

* The average and standard deviation values were calculated from an average of at least four independent stop flow shots.

Overall, the release of EF-G and nucleotide from the two, ribosome trapped, most stable complexes, GDPNP and fus, is much slower in the GDPNP state compared with the fus state (5 and 10 fold respectively, see Table III.1.), suggesting that, upon switch I flipping out, EF-G and GDP are released quasi-simultaneously from the ribosome following GTP hydrolysis.

These functional data complement and strengthen our structural findings, suggesting that post-GTP hydrolysis switch I flipping out correlates with EF-G and nucleotide release from the ribosome. Also, they support a role for switch I dynamics in fusidic acid resistance mechanisms.

III.3. DISCUSSION

The extensive functional and structural studies of EF-G have generated numerous hypotheses on the role of GTP hydrolysis with respect to EF-G's function in translocation. Functional kinetic studies have shown that GTP hydrolysis precedes translocation, and therefore the “on” or “GTP” state of EF-G on the ribosome does not equal its “translocation” state [31]. Comparison with other GTPases, and with EF-Tu in particular, prompted the identification of certain conserved “key” regions within the G domain, which change conformation upon GTP hydrolysis, and in doing so “switch” or “unlock” a universal “spring” mechanism, which couples these localized G domain conformational rearrangements with further structural changes propagated throughout the GTPase molecule.

The specific function and mechanism of action of the switch regions is as yet unknown; however one of the proposed mechanistic hypotheses is that they may convey conformational changes of EF-G which result in decreased contacts with the ribosome, and in turn, in a decreased affinity of EF-G for the ribosome [7-8, 48]. Also, GTP hydrolysis may trigger a biomechanical translocation “unlocking” mechanism [49-50], in which the overall shape of EF-G changes as domains III, IV and V extend and rotate with respect to domains I and II, which remain anchored to the ribosome, causing domain IV of EF-G to act as a “wedge”

in the A site, pushing forward the tRNAs and the mRNA, while at the same time preventing back-translocation [51].

Structural studies of fusidic acid sensitive and resistant mutants [13-15] propose a more detailed explanation of this process by examining the G domain and the switch regions at a molecular level. Thus conformational changes of EF-G start in the G domain at the level of the two switch regions, and subsequently affect the overall shape of EF-G in solution. These rearrangements may be responsible for “unlocking” the inter-domain conformational changes observed by cryo-EM [9]. Our unpublished work regarding switch II interactions with domain III residues situated at the interface between domains I and III (see chapter II) supports these non-exclusive mechanistic hypotheses, while at the same time proposing that GTP hydrolysis, through the switch regions rearrangements, affects not only the overall shape of EF-G and its ribosome contacts, but also the progression of the EF-G cycle from ribosome bound to GTP hydrolysis, and to translocation.

In this study, we examined the role of the switch I region in driving the progression of the EF-G cycle both on and off the ribosome. We investigated the accessibility of switch I to trypsin cleavage in different EF-G states, followed by revealing structural studies which mapped the movement of switch I with respect to the ribosome. By comparing the hydroxyl radical cleavage pattern of the tip of switch I between the pre-hydrolysis and the post-translocational states of ribosome bound EF-G, we found that switch I is projected outward from the ribosome cavity following GTP hydrolysis and 30S translocation.

Our results are supported by studies combining crystallography and cryo-EM analysis of the GDPNP state of EF-G bound to the ribosome [19]. This study found the switch I of *T. thermophilus* EF-G-2 to be unusually ordered and tucked inside a rRNA pocket between helices 14 (30S) and 95 (50S) (Fig. III.16., EF-G-2 GDPNP). Also, the latest crystal structure of EF-G bound to the ribosome in the fus state [20] shows a disordered but outward-oriented, switch I loop (Fig. III.16., EF-G GDP).

Intriguingly, the conformational change we observed for switch I of EF-G is opposite from that reported for the switch I region of EF-Tu, EF-G's "molecular mimic" [52]. In EF-Tu, upon GTP hydrolysis, switch I has been shown to project towards the inter-domain core of EF-Tu [5] (Fig. III.16., EF-Tu GDP) and not outward in solution.

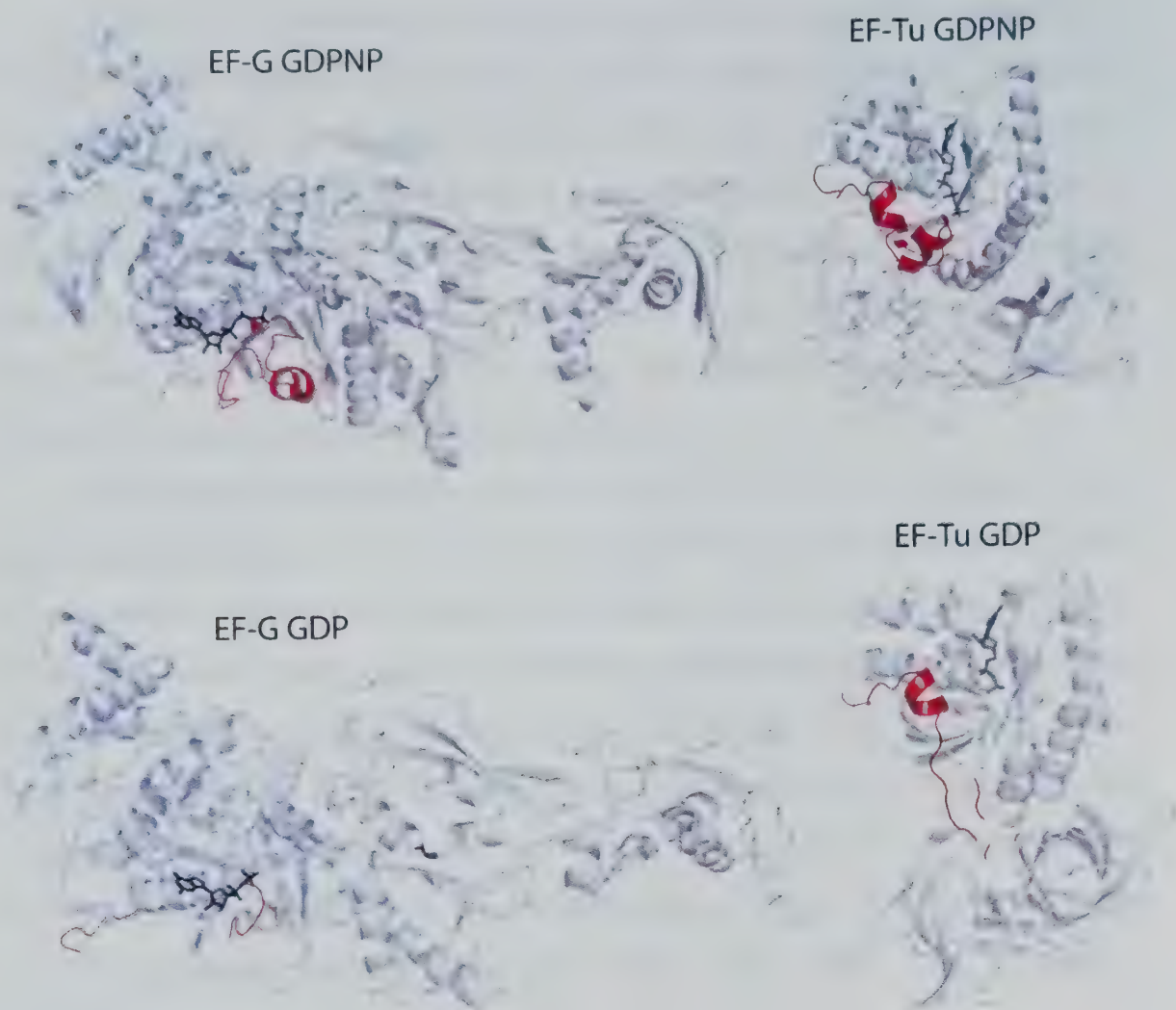


Fig. III.16. Comparative analysis of the switch I conformational changes between the GTP and GDP states of EF-G and EF-Tu

The crystal structures of EF-G in the GTP state (PDB file 1WDT) and in the GDP state (PDB file 2WRI) are shown side by side with crystal structures of EF-Tu in the GTP state (PDB file 1EFT) and in the GDP state (PDB file 1TUI).

EF-G and EF-Tu are both shown in light gray with the conserved switch I region highlighted in red. The bound nucleotides are as indicated and shown in black.

Detailed examination of structural changes encountered in other GTPase proteins, showed that although switch I of EF-G and EF-Tu have a similar GTP (“on”) conformation, they relax in opposite directions upon passage into their GDP (“off”) state (Fig. III.16.), a behavior which is in fact not unheard of among GTPases [1], and which is quite similar with the “divergent” switch I conformation observed in the “off” state of Ras and Ras-related Rho proteins [1].

Cryo-EM studies of EF-G’s eukaryotic homolog, eEF-2, validate our experiments by showing that switch I of eEF-2 becomes disordered upon GTP hydrolysis [17, 53]. On the other hand, cryo-EM studies of EF-Tu show an “off” state with a partially disordered switch I, oriented towards some of the 16S rRNA nucleotides (helices 8 and 14) [54-55]. These very nucleotides were cleaved by 58C-FeBABE in the fus state (Fig. III.10., F.), suggesting that the switch I of EF-G and EF-Tu might, after all, relax into similarly disordered, outward oriented, conformations following GTP hydrolysis.

We further examined the ribosome bound EF-G states in terms of EF-G and nucleotide release from the ribosome. Both GTP and GDP bind to EF-G alone or in complex with the ribosome with similarly high affinities [46-47], while EF-G(free) has a significantly lower affinity for the ribosome compared with the nucleotide complexed state of EF-G [56]. GDPNP has been shown to inhibit EF-G turnover and is in fact released slowly from the ribosome [30, 46, 48]. We observed that both nucleotide and EF-G were released significantly faster from the ribosome in the GDP state compared with the GDPNP state, suggesting that the EF-G-ribosome complex dissociated much faster when EF-G exhibits a flipped-out switch I region.

The antibiotic fusidic acid, which binds to a post-translocational EF-G·GDP·ribosome complex [57] at the interface between domains I and III of EF-G [14, 20], suppresses the release of both EF-G and GDP from the ribosome, and is able to exert its effects even when mixed with EF-G and the ribosome in solution (Fig. III.13. and III.15.). These data suggest that switch I opening may create an entry/binding gate for antibiotic.

Based on our experimental findings as well as on the published structural studies discussed above, we propose the following model (Fig. III.17.) reflecting the functional implications for the conformational changes exhibited by switch I during the EF-G elongation cycle.

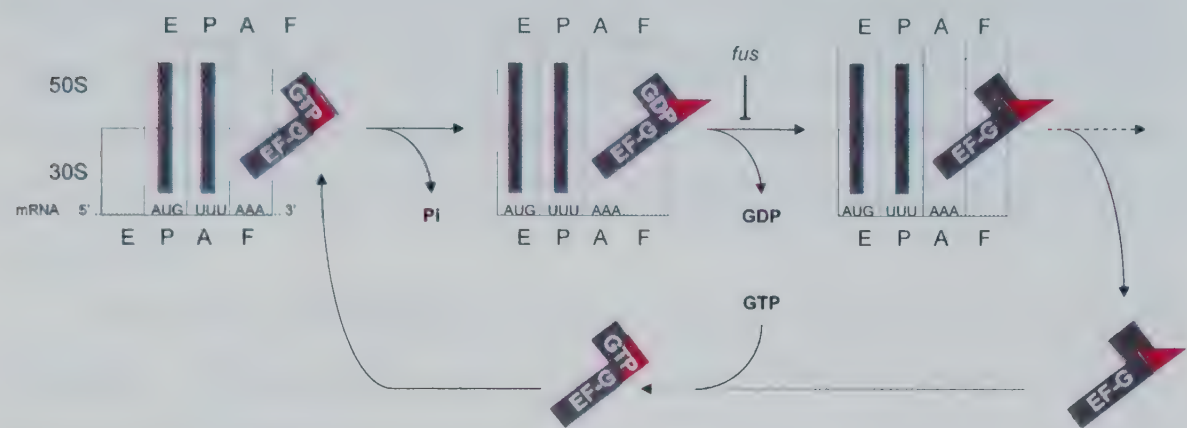


Fig. III.17. A proposed model for nucleotide release and EF-G recycling
 The schematic representations of EF-G and the ribosome are as in Fig. III.1. The switch I region of EF-G is represented as a red triangle, which is shown either flipped in or flipped out with respect to EF-G.

EF-G binds GTP in solution and at that point, switch I becomes partially ordered and tucked inside the G domain (Fig. III.3., A.). Most likely, switch I protects the nucleotide from water attack by interactions with the α phosphate of GTP [19-20]. These interactions are further stabilized upon binding to the ribosome, as switch I becomes protected inside the ribosome cavity, between the G domain of EF-G and helix 95 of the 23S rRNA (Fig. III.3., C. and Fig. III.11., A.). Upon GTP hydrolysis and ribosomal ratcheting, the rearrangements of the G domain may weaken some of the interactions between switch I and the rest of the EF-G molecule, however without causing it to completely flip outward (Fig. III.10., A., 58C-FeBABE - vio state). As the tRNAs and mRNA undergo 30S translocation, and domains III, IV and V of EF-G extend and rotate with respect to domains I and II [9], switch I may lose the remaining hydrogen bonds with the G domain and with the γ phosphate of GTP. It subsequently becomes unanchored and dynamically flopping away from both EF-G and the ribosome (Fig. III.3., C.

and Fig. III.11., A.), and in doing so probably provides an exit pathway for GDP, which in turn facilitates EF-G release from the ribosome. This hypothesis is supported by the observation that GDP release from ribosome-bound EF-G occurs faster than EF-G turnover from the ribosome [46].

Upon release from the ribosome in the (free) state, switch I remains unanchored and exposed in solution (Fig. III.3., A.) until a new GTP molecule becomes bound onto EF-G and causes switch I to close onto it. Unlike EF-Tu, EF-G recycles spontaneously in solution, as GTP predominates in healthy cells over GDP, plus EF-G has been shown to have a slightly higher affinity for GTP than for GDP [46].

In parallel, the ribosome also undergoes conformational changes during elongation. As discussed in chapter II, domain III of EF-G is needed for ribosome activated GTP hydrolysis [58] and structural studies show that domain III contacts both ribosomal subunits [20]. Through these interactions, EF-G may be able to “feel” the unratcheted or ratcheted state of the ribosome and thus self-regulate GTP hydrolysis [20]. Similar feedback based mechanisms have been proposed for translocation [7-8], therefore it is possible that EF-G and the ribosome function synchronously to regulate switch I conformational changes as well.

Our hydroxyl radical probing experiments show switch I flipped in for the GDPNP and vio states and flipped out in the fus state (Fig. III.10.), therefore suggesting that switch I flips outward after GTP hydrolysis and 30S translocation. However, comparative trypsin probing experiments of trapped u and r ribosome complexes (Fig. III.5.) indicate similar exposure of switch I regardless of either 50S or 30S translocation (Fig. III.6., D.), and suggesting that switch I's conformational change is primarily GTP hydrolysis dependent. These results can be reconciled if some of the elongation steps were in fact uncoupled processes between EF-G and the ribosome. Indeed, ribosomal ratcheting and 50S translocation [38, 59] as well as GTP hydrolysis and translocation [30, 40, 46] have been proposed to be decoupled mechanisms. Thus it is possible that certain GTP hydrolysis driven interactions between EF-G and the ribosome may control switch I movements.

III.4. CONCLUSIONS

In this chapter we examined the switch I component of the GTP hydrolysis controlled “spring” mechanism found in the GTPase EF-G. We defined six EF-G states, three in solution and three trapped on the ribosome at different stages during EF-G’s elongation cycle. We then examined these EF-G states in terms of switch I position changes and functional implications in GTP hydrolysis, translocation, and EF-G and nucleotide recycling.

A combination of trypsin and hydroxyl radical probing, as well as functional EF-G and nucleotide release experiments revealed that the switch I region of EF-G changes conformation from a tucked in form, while in the GTP state, to a flipped out form, correlated with GTP hydrolysis and 30S translocation.

These data led us to propose that the conformational change observed in switch I may be correlated with GDP release from EF-G and thereafter with EF-G release from the ribosome.

III.5. FUTURE DIRECTIONS

Our study clearly showed by hydroxyl radical probing that switch I of EF-G flips out after GTP hydrolysis; however, it would be interesting to clarify which processes are affected by switch I flipping out from the ribosome. If the switch I movement were reversibly prevented by locking its tip against domain III, one could determine by detailed functional experiments precisely when in the elongation cycle does switch I flip out and which processes are most affected by its entrapment in the GTP conformation.

The ideal tool for such experiments would be a reversible crosslinking agent, which could immobilize the closed conformation of switch I. Such a crosslinker would have to be highly specific both in terms of the residues chosen to be crosslinked, as well as in terms of its middle arm span length (i.e. its molecule should be ideally under 10-15 Å in order to minimize switch I mobility post-crosslinking).

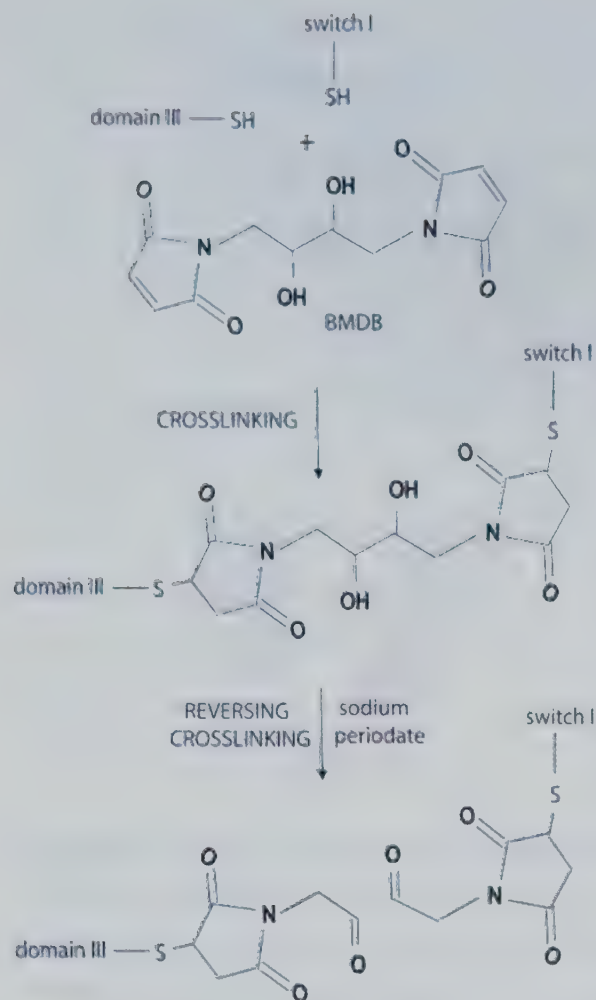


Fig. III.18. The reversible BMDDB crosslinking method

BMDDB (1,4-bismaleimidyl, 2,3-dihydroxybutane) is a sulfhydryl specific, reversible, homobifunctional crosslinking agent, marketed by Thermo Scientific - Pierce Protein Research Products. As shown in Fig. III.18., BMDDB has two reactive maleimide groups, separated by a 10.2 Å long spacer arm, which enable it to create a stable covalent bond between two thiol containing (e.g. cysteine) residues, situated within 10 Å or less of each other. BMDDB reacts under mild conditions of pH (6.5 to 7.5) and conveniently, the crosslink can be reversed in the presence of high concentrations (15 mM) of sodium periodate.

As explained above, we need a double cysteine mutant of EF-G, with one mutation situated at the tip of switch I (58C), and a second mutation situated less than 10 Å away. By examining the position of switch I with respect to domain III in the GDPNP state of EF-G-2 [19] (Fig. III.19., A.), three residues from helix B_{III} of domain III seem to be adequate candidates for mutation to cysteines: 468C, 471C and 478C (all residues in this section are *E. coli* numbers), out of which the first two are the least conserved. We used Pymol modeling to mutate these residues to cysteines and estimate the distances between their sulfhydryl groups and the thiol of 58C. As diagrammed in Fig. III.19., B., all domain III residues considered for mutagenesis, once mutated to cysteines, would be within the range of the BMDDB crosslinker (10.2 Å) from 58C, suggesting that switch I can physically be immobilized against domain III while in its tucked in state.

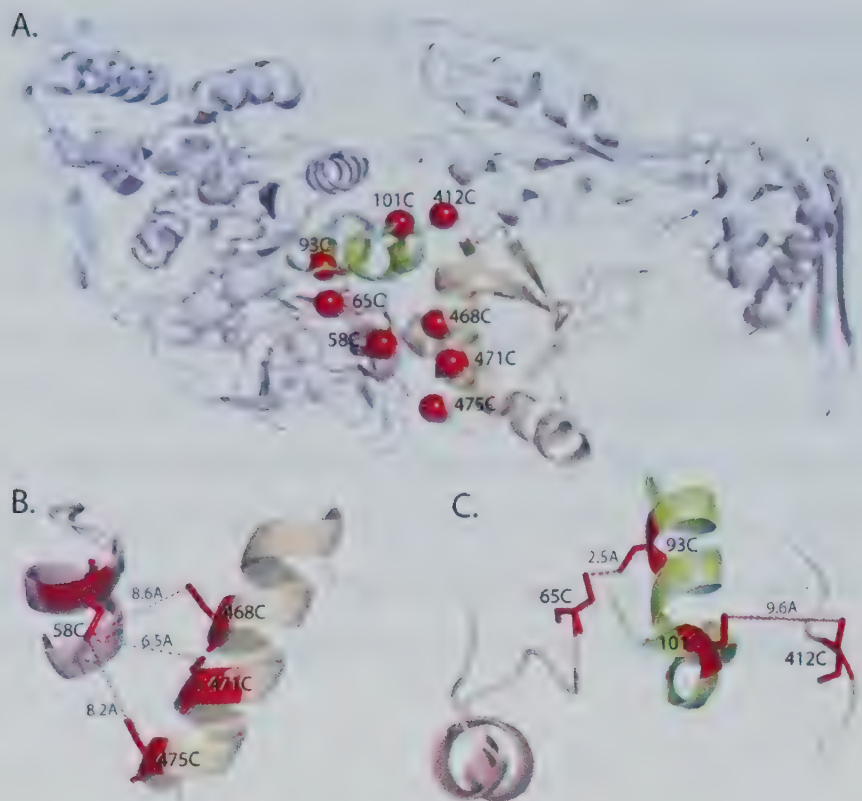


Fig. III.19. The location of 58C with respect to the other proposed mutants

A. EF-G-2 (PDB file 1WDT) is represented in light gray, with switch I in salmon, switch II in limon and domain III in light orange. The cysteine residues are represented with red spheres centered on the C_{α} atoms.

B., C. Detailed view of the interface between switch I and domain III (B) and between switch I, switch II and domain III (C), respectively. The color code from panel A is maintained. The Pymol program was used to mutate all residues to cysteine and to measure the distances between their thiol groups (Å).

As shown in Fig. III.10., 58C-FeBABE cleaves nucleotides 2662 and 2663 in all ribosome bound states, suggesting it is possible to crosslink switch I to domain III in either free(GTP) or (free)GDP EF-G states. The internal crosslinking efficiency could be verified by trypsin probing. As the crosslinking efficiency is not expected to be 100 %, one could purify the intrinsically crosslinked EF-G species from the un-reacted EF-G by hydrophobic interactions chromatography followed by desalting and concentration steps, as in [60].

By comparing the crosslinking product and the wild type protein for functionality in GTP hydrolysis and translocation assays, one could identify which elongation processes proceed with a closed switch I, and which require the presence of the reversing crosslinking agent. It would also be interesting to repeat

our mant-nucleotide release experiments with switch I crosslinked to domain III in the presence of mant-GDPNP and mant-GDP bound to EF-G. These experiments would provide insight into the validity of our functional conclusions that switch I flipping outward from EF-G and the ribosome is correlated with nucleotide release.

If these experiments succeed, we could further expand this technique to immobilize switch I against switch II, and switch II against domain III by using strategically chosen double cysteine mutants (Fig. III.19., A. and C.), e.g. EF-G(65C&93C) and EF-G(101C&412C), respectively. These experiments would provide essential information in terms of the dynamics of the two switch regions with respect to each other and of switch II with respect to domain III, respectively.

III.6. MATERIALS AND METHODS

III.6.1. Reagents, general experimental procedures and buffers

The reagents delineated in chapter II.6.1., purchased from the same sources, were also used for the experiments described in this chapter. In addition, trypsin, fusidic acid and GDPNP were purchased from Sigma Aldrich. Mant-GDPNP and Oregon Green (OG) maleimide were purchased from Invitrogen. Viomycin was a kind gift from Julian Davies (University of British Columbia).

E. coli ribosomes, mRNA and pyrene labeled mRNA oligonucleotide were obtained exactly as described in chapter II.6. *N*-Ac-Phe-tRNA^{Phe} was purified by Kevin Wilson (over 90 % purity) as described [61].

Throughout this chapter, the GDPNP and fus complexes were formed by directly mixing the pre-formed ribosomal complexes with nucleotide and EF-G, while the vio state was formed by incubating the pre-translocational ribosomal complex for 10 minutes at 20° C with viomycin, followed by subsequent addition of EF-G and GTP.

Buffers used exactly as in chapter II are numbered 1 to 19, buffers used only in chapter III are numbered from 20 to 23. Unless otherwise indicated, the

experiments conducted in this chapter were done in buffer 20. **Buffer 20:** 80 mM HEPES-KOH pH 7.7, 50 mM NH₄Cl, 10 mM MgCl₂ and 1 mM DTT; **buffer 21:** 60 mM HEPES-KOH pH 7.7, 80 mM KCl and 9.5 mM MgCl₂; **buffer 22:** 80 mM K-borate pH 8.1, 10 mM MgCl₂, 100 mM NH₄Cl and 6 mM β -mercaptoethanol; **buffer 23:** 10 mM HEPES-KOH pH 7.0 and 100 mM KCl.

III.6.2. EF-G mutagenesis and purification

The plasmid encoding hexahistidine tagged wild type *E. coli* EF-G was constructed as described in II.6.3. The hexahistidine tagged cysteineless version of this plasmid, as well as EF-G(196C) and EF-G(426C) were constructed previously [45]. Both plasmids and the above mentioned cysteine mutants were constructed by Kevin Wilson.

The EF-G(58C) mutant was created especially for this study by Melanie Desrosiers, following the mutagenesis technique described in II.6.3. and using the following primer: 5'-GGT AAT ACC ACG ACA CTG CTC CTG CTC C-3'.

Wild type EF-G, EF-G(58C), EF-G(196C) and EF-G(426C) were purified by Roxana Nechifor and Boray Nguyen as described in chapter II.6.4., except the second, size exclusion, purification step was eliminated. The imidazole gradient elution products were dialyzed overnight at 4° C against buffer 7 (see II.6.1.) and stored at - 20° C until further use.

III.6.3. GTP hydrolysis assay

GTP hydrolysis assays for non-FeBABE labeled proteins were conducted by Boray Nguyen as described in II.6.7., with the following modifications. Single turnover conditions (10 μ l final volume) were 1 μ M 70S, 0.8 μ M EF-G and 0.5 μ M GTP (with trace [γ ³²P]-GTP), while multiple-turnover conditions (10 μ l final volume) were 0.5 μ M 70S, 0.04 μ M EF-G and 50 μ M GTP (with trace [γ ³²P]-GTP). In both cases, the complexes were probed with 100 μ M GDPNP (GDPNP state), 250 μ M viomycin (vio state) and 200 μ M fusidic acid (fus state).

The FeBABE-labeled proteins were assayed for GTP hydrolysis under the multiple turnover conditions described above.

III.6.4. Toeprinting and pyrene based translocation assays

Translocation assays were as described in II.6.11 and II.6.12., with the following modifications. Toeprinting involved formation of pre-translocational ribosomal complexes with 1 μ M primer : 0.5 μ M mRNA, 1 μ M 70S, 1.2 μ M tRNA^{fMet} (30 minutes at 37° C), bound to the P site and 1.2 μ M tRNA^{Phe} (20 minutes at 37° C), bound to the A site. Where indicated, 1.2 μ M *N*-Ac-Phe-tRNA^{Phe} (20 minutes at 37° C) was bound to the P site. The assembly of the vio state pyrene based fluorescent translocation complexes (done by Roxana Nechifor) pre-incubated the pre-translocational complex with 0.5 mM viomycin for 15 minutes at 20° C.

In both assays, the pre-translocational complexes were probed in presence of 1 μ M EF-G and 1mM GTP (GTP state), 1 mM GDPNP (GDPNP state), 0.5 mM viomycin (pre-incubated as described above) with 1 mM GTP (vio state), and 1 mM fusidic acid with 1 mM GTP (fus state) respectively.

III.6.5. Trypsin probing assay

All trypsin probing experiments were done by Roxana Nechifor. The free EF-G states were formed in 20 μ l reactions, for 10 minutes at 20° C with 2 μ M wild type EF-G and 0.5 mM GTP (GTP(free) state), GDP (GDP(free) state) or no nucleotide ((free) state). The complexes were then incubated with 4.5 μ g/ml trypsin at 20° C. 2 μ l aliquots were removed at different time intervals, quenched with 340 μ g/ml trypsin inhibitor, denatured for 5 minutes at 90° C in SDS sample buffer (see II.6.1.) and analyzed by 10-12 % SDS-PAGE.

The ribosome-bound EF-G states were formed were formed in 60 μ l reactions, using pre-assembled pre-translocational ribosomal complexes containing: 2 μ M ribosomes, 2.4 μ M phage T4 gene 32 mRNA, 2.4 μ M tRNA^{fMet} (30 minutes at 37° C), bound to the P site, 2.4 μ M tRNA^{Phe} (20 minutes at 37° C), bound to the A site. Where indicated, 2.4 μ M *N*-Ac-Phe-tRNA^{Phe} (20 minutes at

37° C) was bound to the P site. There equal volumes (20 µl each) removed from the pre-translocational ribosome complex were combined with 1.7 µM EF-G as well as 0.5 mM GDPNP (GDPNP state), 0.25 mM viomycin with 0.5 mM GTP (vio state), and 0.5 mM fusidic acid with 0.5 mM GTP (fus state). All reactions were allowed to proceed for 15 minutes at 37° C and then transferred to ice for 5 minutes.

EF-G bound complexes were then purified of free EF-G by filtration (also see II.6.13.): complexes were diluted in 500 µl ice cold buffer 20 (supplemented, as appropriate, with 20 µM fusidic acid or 100 µM viomycin), filtered for 5 minutes under centrifugation at 13,800 x g using microcons YM-100, followed by concentration under vacuum to a volume of 10 µl. The filtered complexes were further denatured with trypsin as described above. In some, experiments, as indicated, a second filtration step was employed at this point. Final samples were denatured for 5-10 minutes at 90° C in SDS sample buffer (see II.6.1.) and analyzed by 10-12 % SDS-PAGE.

III.6.6. Chemical probing of the ribosome

III.6.6.1. Hydroxyl radical probing of rRNA: FeBABE was formed by incubating 10 mM BABE (solubilized in DMSO) and 9 mM FeSO₄ in the presence of 0.1 mM NaOAc pH 6.0 for 1 hour at 20° C. The reaction was then quenched by 10 minutes incubation at 20° C with 10 mM EDTA.

The FeBABE attachment to EF-G single cysteine mutants was done by incubating 10,000 pmols FeBABE with 3000 pmols EF-G for 15 minutes at 37° C in buffer 21. In order to remove the excess un-reacted Fe-BABE, the reactions were then diluted to 500 µl with ice cold buffer 21 and filtered through YM-30 microcons (see II.6.1.) by 8-10 minutes centrifugation at 13,800 x g (repeated three times). The concentration of EF-G-FeBABE proteins was determined by A_{280nm} using an extinction coefficient of 64,282 M⁻¹ · cm⁻¹.

The pre-translocational ribosomal complexes were pre-assembled using 1 µM ribosomes, 2 µM gene 32 mRNA, 2 µM tRNA^{fMet} (30 minutes at 37° C), bound to the P site, and 2 µM tRNA^{Phe} (20 minutes at 37° C), bound to the A site.

The ribosome bound EF-G states were formed for 30 min at 37° C by incubating pre-assembled pre-translocational ribosomal complexes (from above) with 2 μ M FeBABE-labeled EF-G and 1 mM GDPNP (GDPNP state), 1 mM viomycin with 0.5 mM GTP (vio state), and 1 mM fusidic acid with 0.5 mM GTP (fus state), respectively. The complexes were then stabilized by 10 minutes incubation on ice.

Hydroxyl radical probing of the above EF-G states was done for 10 minutes on ice in presence of 0.1 % hydrogen peroxide and 10 mM ascorbic acid. Quenching was done with 160 mM thiourea, followed by ethanol precipitation (1 hour at - 20° C) using 1/10 (v/v) NaOAc pH 5.2 and 3X (v/v) 100 % ethanol. The pelleted RNA and protein products were then resuspended in buffer 13 (see II.6.1.) and the proteins were removed by phenol pH 6.6 and chloroform extractions (repeated six times). The extracted RNA was then precipitated with 3X (v/v) 100 % ethanol for 30 minutes at 20° C. The pellets were washed with 70 % ethanol, dried for 2-3 minutes by centrifugation under vacuum, resuspended in 125 μ l np H₂O (final RNA concentration was approximately 0.16 μ M) and stored at - 80° C until further use.

III.6.6.2. DMS and kethoxal modification of rRNA: A vacant (v) ribosome·mRNA complex containing 2 μ M ribosomes and 2.4 μ M mRNA was incubated for 20 minutes at 37° C with 2.4 μ M *N*-Ac-Phe-tRNA^{Phe} to obtain an unratcheted (u) ribosomal complex. The ratcheted (r) complex was obtained by incubating the u complex with 1 mM puromycin for 30 minutes at 37° C.

The three ribosomal complexes (v, u, and r) were probed with DMS as described in II.6.13., except the complexes were formed in buffer 20 with 1.7 μ M EF-G in the presence of 0.5 mM GTP and 1 mM fusidic acid.

The kethoxal RNA probing was done following the protocol described in [43], with some modifications. The complexes were formed in the fus state, as for the DMS probing, in buffer 22. The reactions were quenched by addition of 0.1 mM NaOAc pH 6.5 and 333 mM K-borate pH 7.0, followed by precipitation (as described for DMS). The pellets were resuspended in buffer 13 (see II.6.1.) with 50 mM K-borate pH 7.0. The rest of the RNA purification procedure was done as described for the DMS modification experiment (see II.6.13.).

III.6.6.3. Primer extension analysis of the hydroxyl radical, DMS and kethoxal probing of the rRNA was done exactly as described in II.6.13.

III.6.7. EF-G (OG) release assay

EF-G(142C) Oregon Green (OG) labeling and purification was done by Boray Nguyen. YM-30 microcons (see II.6.13.) were used to exchange buffer 7 (see II.6.1.) for buffer 23. 100 μ M EF-G(142C) was incubated overnight in the dark, at 20° C, with 500 μ M OG and limiting β -mercaptoethanol (25 μ M). The labeling reaction was quenched with 100 mM β -mercaptoethanol. Properly-folded EF-G(OG) was isolated in buffer 23 by size exclusion chromatography using a Superdex200 (10/300) column attached to an ÄKTA purifier. The fractions containing OG-labeled EF-G were pooled together, dialyzed in the dark overnight at 4° C against buffer 7, and stored at - 80° C until further use. The ratio, “*R*”, of OG attached to EF-G was determined using the $A_{280\text{nm}}$ and $A_{496\text{nm}}$ using the equation: $R = (A_{496} \times \epsilon_{\text{EF-G}}) / (A_{280} \times \epsilon_{\text{OG}})$, where ϵ is the extinction coefficient for OG ($\epsilon_{\text{OG}} = 81,000 \text{ M}^{-1}\text{cm}^{-1}$) and EF-G ($\epsilon_{\text{EF-G}} = 64,282 \text{ M}^{-1}\text{cm}^{-1}$), respectively. The calculated *R* of OG attached to EF-G was between 0.6 and 0.8.

To measure EF-G release from different ribosomal complexes (Fig. III.12., A., III.13., A.), 2 μ M EF-G(426C-OG) and 3 μ M ribosomes were incubated for 10-15 minutes at 20° C with 0.2 mM GDPNP (GDPNP state), 0.1 mM viomycin and 0.2 mM GDP (vio state) or with 0.1 mM fusidic acid and 0.2 mM GDP (fus state). By using the rapid mixing apparatus, these complexes were rapidly mixed with 20 μ M wild type EF-G.

In the experiment shown in Fig III.14., 2 μ M EF-G(426C-OG), 3 μ M 70S, 10 μ M GDP and 0.1 mM viomycin were rapidly mixed with either 10 μ M wild type EF-G or a mixture of 10 μ M wild type EF-G and 1 mM GDP.

In the experiment shown in Fig. III.15., A., 2 μ M EF-G(426C-OG), 3 μ M 70S and 0.2 mM GDP were rapidly mixed with either 20 μ M wild type EF-G or a mixture of 20 μ M wild type EF-G and 0.1 mM fusidic acid.

After mixing, the fluorescence increase was obtained by exciting OG at 492 nm and collecting its emission spectra at 518 nm. The data were fitted to a

single exponential rise equation: $F_t = F_0 + A \times [1 - \exp(-k_d \times t)]$, where F_0 and F_t are the fluorescence intensities (a.u.) at times 0 and t respectively, A is the amplitude of the fluorescence change, while k_d is the dissociation rate constant.

III.6.8. Mant-GDPNP/GDP release assay

To measure nucleotide release from different ribosomal complexes (Fig. III.12., B., III.13., B.), 2 μ M wild type EF-G and 3 μ M ribosomes were incubated with 2 μ M mant-GDPNP (GDPNP state), with 0.1 mM viomycin and 2 μ M mant-GDP (vio state) or with 0.1 mM fusidic acid and 2 μ M mant-GDP (fus state). These complexes were then rapidly mixed with 2.4 mM of the corresponding unlabeled nucleotide (GDPNP or GDP).

In the experiment shown in Fig. III.15.,B., 2 μ M wild type EF-G, 3 μ M 70S and 2 μ M mant-GDP were rapidly mixed with either 2.4 mM GDP or a mixture of 2.4 mM GDP and 0.1 mM fusidic acid.

After mixing, the fluorescence decrease was obtained by exciting the mant group at 362 nm and collecting its emission spectra at 444 nm. The data were fitted to a single exponential decay equation: $F_t = F_0 + A \times \exp(-k_d \times t)$, where F_0 and F_t are the fluorescence intensities (a.u.) at times 0 and t respectively, A is the amplitude of the fluorescent change, while k_d is the dissociation rate constant.

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**IV. EF-P ACTS AT THE BORDERLINE BETWEEN TRANSLATIONAL
INITIATION AND ELONGATION BY STABILIZING THE INITIATOR
TRNA IN THE P SITE**

IV.1. INTRODUCTION

E.coli translational systems have been extensively studied *in vitro* due to their relative simplicity in terms of efficient polypeptide chain synthesis. The PURE (protein synthesis using recombinant elements) system, containing, in addition to ribosomes, three IFs (IF-1, IF-2, and IF-3), three EFs (EF-Tu, EF-Ts, and EF-G), four termination factors (RF-1, RF-2, RRF, and RF-3), 20 aminoacyl-tRNA synthetases, 20 amino acids, methionyl-tRNA transformylase, T7 RNAP, 46 tRNAs, ATP, GTP, creatine phosphate, 10-formyl-5,6,7,8-tetrahydrofolic acid, creatine kinase, myokinase, nucleoside-diphosphate kinase and pyrophosphatase, is able to sustain effective protein synthesis *in vitro* [1]. In living cells however, other factors may come into play to modulate optimal protein synthesis [2].

One of them is EF-P (Fig. IV.1.), a small 20.6 kDa protein, which, by its “L” shape and its negatively charged surface, is reminiscent of a tRNA [3]. *In vivo* studies showed that the *efp* gene [4] is conserved throughout eubacteria [2, 5] and is essential for viability and protein synthesis in *E. coli* [4].

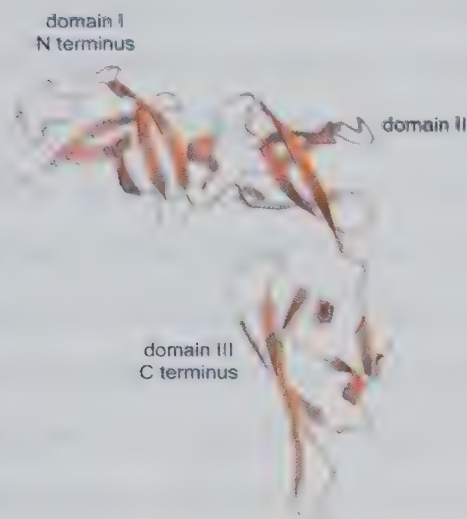


Fig. IV.1. EF-P crystal structure [3]
(PDB file 1UEB)

EF-P consists of three β -barrel domains which form a tRNA-like shape, with two arms (65 Å and 53 Å long) oriented at an angle of 95° from each other. Domain I mimics the acceptor end, while domain III mimics the anticodon loop, of a tRNA. Domain II has an oligonucleotide-binding fold and was hypothesized to interact with the rRNA.

EF-P has a theoretical isoelectric point of approximately 4.5, and is believed to function as a stable monomer under physiologic ionic conditions and within a wide range of temperature (24° C to 40° C) [6]. EF-P’s binding affinity for the ribosome is quite low (K_M is close to 10 μ M) [7] and yet 2D gel quantification studies showed that, regardless of the stage of cell growth, there is

approximately one EF-P molecule per 10 ribosomes in the cytoplasm of an *E. coli* cell [8], which translates to one tenth of the amount of EF-G and about 1 % of the amount of EF-Tu. Thus, since its low ribosome affinity is not compensated by an increased amount, it was postulated that EF-P may not act ubiquitously during elongation like EF-G or EF-Tu, which associate cyclically up to 20 times per second with the ribosome during elongation [9], but rather at a specific step in translation [10]. Further studies emphasized that EF-P, although initially characterized as an elongation factor, bears no functional similarity with the other elongation GTPases, does not bind GTP or GDP, and cannot substitute for the activity of EF-Tu or EF-G on the ribosome [6].

EF-P was first characterized in 1975 by its ability to stimulate by 10 fold the peptidyltransferase activity of *E. coli* ribosomes between the antibiotic puromycin and the initiator fMet-tRNA^{fMet} [7], hence the name of “elongation factor P” (P - **p**eptidyltransferase). Puromycin mimics the 3' end of a charged tRNA and terminates protein synthesis by becoming irreversibly incorporated into the growing polypeptide chain. Further puromycin-based studies proposed that EF-P may act to preferentially stimulate the formation of certain first peptide bonds over others, favoring A site binding and polypeptide chain elongation for tRNA fragments charged with small and/or hydrophilic aminoacyl moieties [10]. Similar studies investigated EF-P's effect on the translation of the ribohomopolymers poly-U (encoding for poly-Phe) and poly-A (encoding for poly-Lys) using Phe-tRNA^{Phe} and Lys-tRNA^{Lys}, as well as EF-G and EF-Tu [6]. These studies showed that although EF-P does stimulate poly-Lys over poly-Phe formation, in the absence of EF-P, poly-Phe forms spontaneously faster even than EF-P enhanced than poly-Lys synthesis. Such data supports the puromycin based findings that certain acceptor aa-tRNAs may require additional help for incorporation into the polypeptide chain [6-7].

Intrigued by its tRNA-like shape and charge, as well as by its stimulatory effects on peptide bond formation *in vitro*, numerous functional studies have attempted to identify EF-P's binding site on the ribosome. Antibiotic sensitivity studies [6, 11-12] have shown that EF-P seems to be most inhibited by PTC

inhibitors (chloramphenicol and lincomycin) and by initiation complex formation inhibitors (streptomycin, oxazolidinones). Ribosome deletion and reconstitution studies seemed to support P or A site affinity for EF-P, since L16, a protein shown to influence PTC activity [13], is required for EF-P mediated peptide bond synthesis [14-16], while L27, a protein influencing A site binding [17] has been shown to stimulate fMet-puromycin peptide bond formation to a similar extent as EF-P [18]. Also, RNA modification studies [19] showed EF-P protection in the A/P site of both ribosomal subunits, thus it was proposed that EF-P might bind to the A, P or A/P site(s).

However, a recent 3.5 Å resolution crystal structure of EF-P bound to the ribosome with fMet-tRNA^{fMet} (P site) and mRNA places EF-P in between the ribosomal P and E sites (P/E site) [20]. EF-P is shown here bound in between the P site fMet-tRNA^{fMet} and the E site r-protein L1. Domains I and III of EF-P are adjacent to the initiator tRNA's CCA end and anticodon loop respectively, while domain II of EF-P is held in place by tight interactions with the stalk of L1 [20]. L1 has been shown to function as an exit gate for the E site tRNA, by adopting "open" or "closed" conformations depending on ribosomal subunits ratcheting [21-22] and E site occupancy [23-24]. When the ribosome is in the "classical" position (unratcheted) with an empty E site, the L1 stalk is unanchored and flips between "open" and "closed" conformations. Upon peptide bond formation and ribosomal ratcheting, and in the presence of a deacylated tRNA in the E/E position, L1 adopts a "closed" conformation, in which its stalk reaches inside the inter-subunit cavity and makes contact with the elbow of the deacylated tRNA [25-27], much like a "guided exit" or "pullout" mechanism. A similarly "closed" conformation of L1 and similar contacts of its stalk with domain II of EF-P (which has been proposed to mimic the elbow of a tRNA [3]) have been described by the recent crystal structure of EF-P on the ribosome [20]. In fact, the entire EF-P molecule has been proposed to structurally mimic an uncharged tRNA [3], domain I being the CCA end, domain II - the elbow region, and domain III - the anticodon loop (Fig. IV.2.). Therefore the interaction between the L1 stalk and domain II of EF-P may be part of the exit mechanism of this protein.

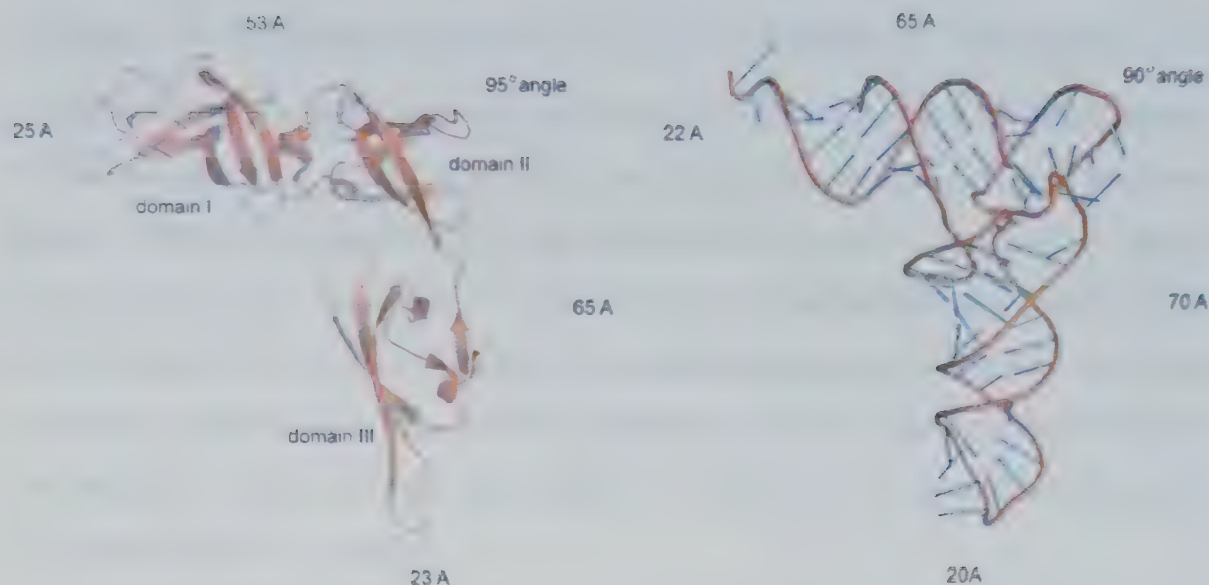


Fig. IV.2. Structural comparison between EF-P and tRNA^{Phe}

Shown here are the crystal structures of *T. thermophilus* EF-P (PDB file 1UEB) (right) and *Saccharomyces cerevisiae* (*S. cerevisiae*) tRNA^{Phe} (PDB file 1EVV) (left). The length and width of each arm (Å) and the inter-arms angles (°) are indicated.

Since its discovery, the hypothesis that EF-P catalytically influences the formation of the first peptide bond [7] has not been challenged. However, recent crystallographic evidence shows that although EF-P is tightly bound to the P/E site of the ribosome and makes contact with the P site initiator tRNA, neither the conformations of the PTC or of the P site are affected by its presence [20]. This observation, combined with its scarce presence in the cell [8] and its low affinity to the ribosome [7], suggests that in fact EF-P indirectly promotes peptide bond formation, by correctly orienting and stabilizing the initiator tRNA in the P site [20]. Such a hypothesis is not unreasonable, since similar roles have already been attributed to r-proteins L16 and L27, in terms of stabilizing the PTC and the binding of the A site tRNA, respectively [13].

Archaeobacteria and eukaryotes lack EF-P but have an analogous factor, eIF-5A, which, although not essential for cell viability [28], shares 64 to 84 % sequence and structural similarity with domains I and II of bacterial EF-P [3, 29] and stimulates the formation of the initial peptide bond between fMet-tRNA^{fMet} and puromycin [30-31]. eIF-5A contains the unusual and unique amino acid

hypusine (*N* ϵ -4-amino-2-hydroxybutyl lysine), formed by post-translational modification of the side chain of a lysine residue, a modification of which seems to activate eIF-5A's ability to associate with the ribosome [32-33] and promote peptide bond synthesis [34-35]. The hypusinated lysine is part of a larger conserved sequence, which can be traced all the way to the bacterial kingdom [2], and although there is some evidence that the corresponding *E.coli* EF-P lysine undergoes a similar post-translational modification in [19], the gene encoding for the enzyme which catalyzes hypusination, deoxyhypusine synthetase, has not been identified in *E. coli* [36].

Despite being catalogued as an initiation factor, eIF-5A has also been linked to mRNA synthesis, stabilization and decay [28, 37-38], nucleocytoplasmic transport of the HIV-1 Rev protein [39-40], and to oncogenic pathways in humans [41]. Furthermore, recent studies infer a possible interaction between eIF-5A and EF-2 [32] and show that the depletion of eIF-5A in *S. cerevisiae* leads to increased ribosomal transit time and polysome accumulation [42-43], thus raising a very distinct possibility that eIF-5A might be primarily an elongation factor.

We proposed to investigate EF-P role in translation by assessing at what stage during protein synthesis EF-P comes into play. Given its striking structural similarities with eIF-5A [3], we also wondered whether all three domains of EF-P serve equally important roles in terms of EF-P interaction with the ribosome, and in terms of EF-P function. We cloned the hexahistidine tagged version of EF-P and probed its activity in presence of initiation and elongation factors, especially EF-G. We then looked at the importance of well conserved residues throughout all three domains of EF-P by mutating them to alanines and assessing their ability to stimulate the formation of the first peptide bond.

We found that EF-P is not involved in initiation complex formation and has no functional correlation with EF-G, suggesting that EF-P probably acts at an intermediate step between initiation and elongation. Our mutagenesis studies show that domain III of EF-P is of most importance for ribosome binding, while the tip of domain I is probably involved in making essential contacts with the initiator tRNA and thus promote the formation of the first peptide bond formation.

IV.2. RESULTS

IV.2.1 The puromycin-based peptide bond formation assay

The puromycin assay was used to first characterize EF-P as a peptidyltransferase catalyzing factor [7]. Puromycin is an amino nucleoside antibiotic derived from *Streptomyces alboniger*, which mimics the 3' end of Tyr-tRNA^{Tyr}, except its adenine group has a double methylated N₆, the ester bond linking the ribose sugar and the tyrosyl moiety is replaced by an amide bond (red), and the tyrosyl moiety has a para-methoxy instead of a para-hydroxyl group attached to the benzene ring (Fig. IV.3.).

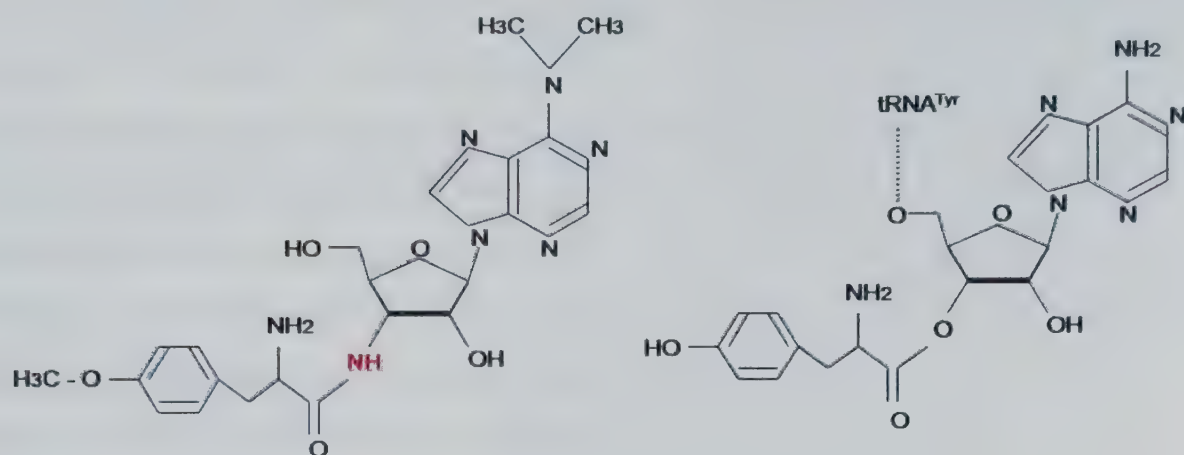


Fig. IV.3. Comparison between puromycin and Tyr-tRNA^{Tyr}

The chemical structures of puromycin (3'-deoxy-*N,N*-dimethyl-3'-(*O*-methyl-L-tyrosinamido)adenosine), right, and of the 3' end of a Tyr-tRNA^{Tyr}, left, are shown above. The tRNA structure is interrupted after the 3' adenosine and indicated as "...tRNA^{Tyr}". The critical amide bond of puromycin is shown in red.

As puromycin grossly resembles only the aminoacyl moiety attached to the very last 3' adenosine of a tRNA, the two molecules differ by approximately 25 kDa in molecular weight. Due to its small size, puromycin easily binds to the ribosomal A site; however, since it lacks the anticodon arm of a real tRNA, it bypasses tRNA decoding and accommodation mechanisms. Upon A site binding, puromycin forms a non-hydrolysable amide bond instead of the normal ester linkage between the A site aa-tRNA and the P site polypeptide chain, which renders the puromycin peptidyltransferase product unable to further elongate. As a

result, the puromycin bound peptide is released from the ribosome and protein synthesis is prematurely terminated. Due to its ability to strongly bind growing polypeptide chains, while bypassing A site accommodation and decoding mechanisms, which are in fact rate limiting steps to the peptidyltransfer reaction, puromycin is a widely used tool for assessing the kinetics of peptide bond formation deep in the core of the PTC.

We used an improved version of the puromycin assay from that originally described in [7, 10] to investigate the kinetics of peptide bond formation between either $f[^{35}\text{S}]\text{Met-tRNA}^{\text{fMet}}$ or $\text{Phe-tRNA}^{\text{Phe}}$ and puromycin. First, we used a full length mRNA instead of the AUG fragment used in [7]. Our mRNA is a fragment (128 nucleotides long) of the gene 32 product from phage T4. It contains a SD sequence, which aids in the correct positioning of the AUG codon into the P site. The following codon after AUG encodes for phenylalanine (UUU), which is also important for our experiments, since we wanted to look at more than just the formation of the very first peptide bond. Secondly, we used only full length tRNAs, charged by aa-tRNA synthetases found in the S100 fraction. The charged tRNA fragments used in [10] contained only the aminoacylated 3' CCA end of a tRNA, rendering these molecules impervious to natural accommodation, binding and hybrid state formation mechanisms. Thirdly, we used 70S ribosomes instead of isolated 30S or 50S subunits as in [10].

Upon assembly of initiation complexes with 70S, mRNA and $f[^{35}\text{S}]\text{Met-tRNA}$, the initiator tRNA would bind to the P site. Puromycin would then react with the P site tRNA and form $f[^{35}\text{S}]\text{Met-puromycin}$ (Fig. IV.4., A.), which is released from the ribosome.

In order to measure the kinetics of the peptidyltransfer reaction between puromycin and phenylalanine, we started by forming initiation complexes as above. We then bound $\text{Phe-tRNA}^{\text{Phe}}$ to the A site, in accordance with the next UUU codon in the mRNA. EF-G, in the presence of GTP, would then translocate the $f[^{35}\text{S}]\text{Met-Phe-tRNA}^{\text{Phe}}$ product to the P site and the deacylated $\text{tRNA}^{\text{fMet}}$ to the E site, thus clearing the A site. Upon addition of puromycin, one can measure

the kinetics of f[³⁵S]Met-Phe-puromycin formation (Fig. IV.4., B.) after its release from the ribosome.

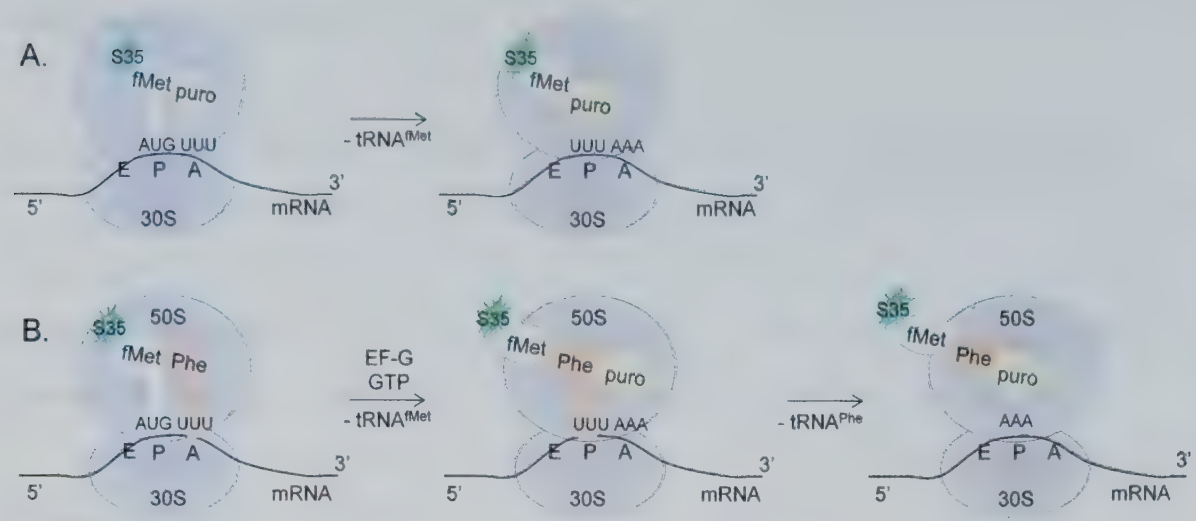


Fig. IV.4. Designing a modified puromycin assay to study and compare the kinetics of peptide bond formation between different substrates

A. f[³⁵S]Met-puromycin peptide bond formation is assessed upon addition of puromycin to pre-formed ribosomal complexes containing f[³⁵S]Met-tRNA^{fMet} in the P site.

B. Phe-puromycin peptide bond formation is assessed by addition of puromycin to pre-formed ribosomal complexes containing f[³⁵S]Met-Phe-tRNA^{Phe} in the P site.

We have verified the functionality of our assays by numerous controls. We calculated the charging efficiency of our tRNAs to be over 90 % (see IV.6.5.). We verified that the 1M NaOH indeed quenches the peptidyltransfer reactions, as we have combined the same quenched samples with scintillation counter fluid and quantified the formation of f[³⁵S]Met-puromycin at 12 hour intervals; we found no difference between samples. We also found that either tRNA, charged or uncharged, does not go into the ethyl acetate phase. Thus no tRNA bound [³⁵S]Met (either f[³⁵S]Met-tRNA^{fMet} or f[³⁵S]Met-Phe-tRNA^{Phe}) contaminated our measurements. Also, we have verified the activity of our EF-G preparation in terms of GTP hydrolysis and translocation, and found it to be fully active (see Fig. IV.8.). We have also verified the integrity and purity of our mRNA and charged tRNAs by 6 % 7 M urea PAGE, and found them to have migrated

correspondingly to their determined molecular weights, with only a few contaminating degradation fragments present in the mRNA preparation.

We can therefore conclude that both our assays indeed undergo the steps described in Fig. IV.4.

IV.2.2. The hexahistidine tagged EF-P is functional in puromycin assays

We cloned the *E. coli efp* gene expressing a C-terminal hexahistidine tag EF-P (see IV.6.2.). The protein was purified by Ni-NTA affinity chromatography, followed by anion exchange chromatography. This double step purification procedure yielded a roughly 99 % pure EF-P preparation (Fig. IV.5., A.).

In order to verify the activity of our EF-P construct, we attempted to reproduce previously reported effects of EF-P on peptide bond formation [7]. We examined the EF-P concentration dependency of $f[^{35}\text{S}]\text{Met-puromycin}$ peptide bond formation (Fig. IV.5., B.), and found EF-P to have an associated K_M of approximately 10 μM , coupled with maximal stimulation of the formation the first peptide bond by roughly 10 fold (0.075 pmols of $f[^{35}\text{S}]\text{Met-puro min}^{-1}$ in the absence of EF-P and 0.54 pmols min^{-1} in the presence of EF-P; see Table IV.1.). These results are in agreement with previously calculated values [7], thus our EF-P protein is functional at the same levels as the wild type EF-P used in previous studies.

It has been reported that EF-P activity in terms of peptidyltransferase stimulation is dependent on the hydrophobicity and the size of the aminoacyl moiety attached to the A site tRNA, e.g. the bulky highly hydrophobic Phe-tRNA^{Phe} would receive the least stimulation from EF-P for incorporation into the polypeptide chain [10]. In order to further verify EF-P's ability to stimulate certain peptide bonds over others, we decided to compare EF-P's effect over $f[^{35}\text{S}]\text{Met-puromycin}$ formation with that exerted over Phe-puromycin product formation. We used the assay template described in Fig. IV.4.B. Indeed, in the presence of EF-P, we observed a stronger stimulation of the rate of $f[^{35}\text{S}]\text{Met-puromycin}$ formation compared with the rate of Phe-puromycin peptidyltransferase reaction (Fig. IV.5.,C.).

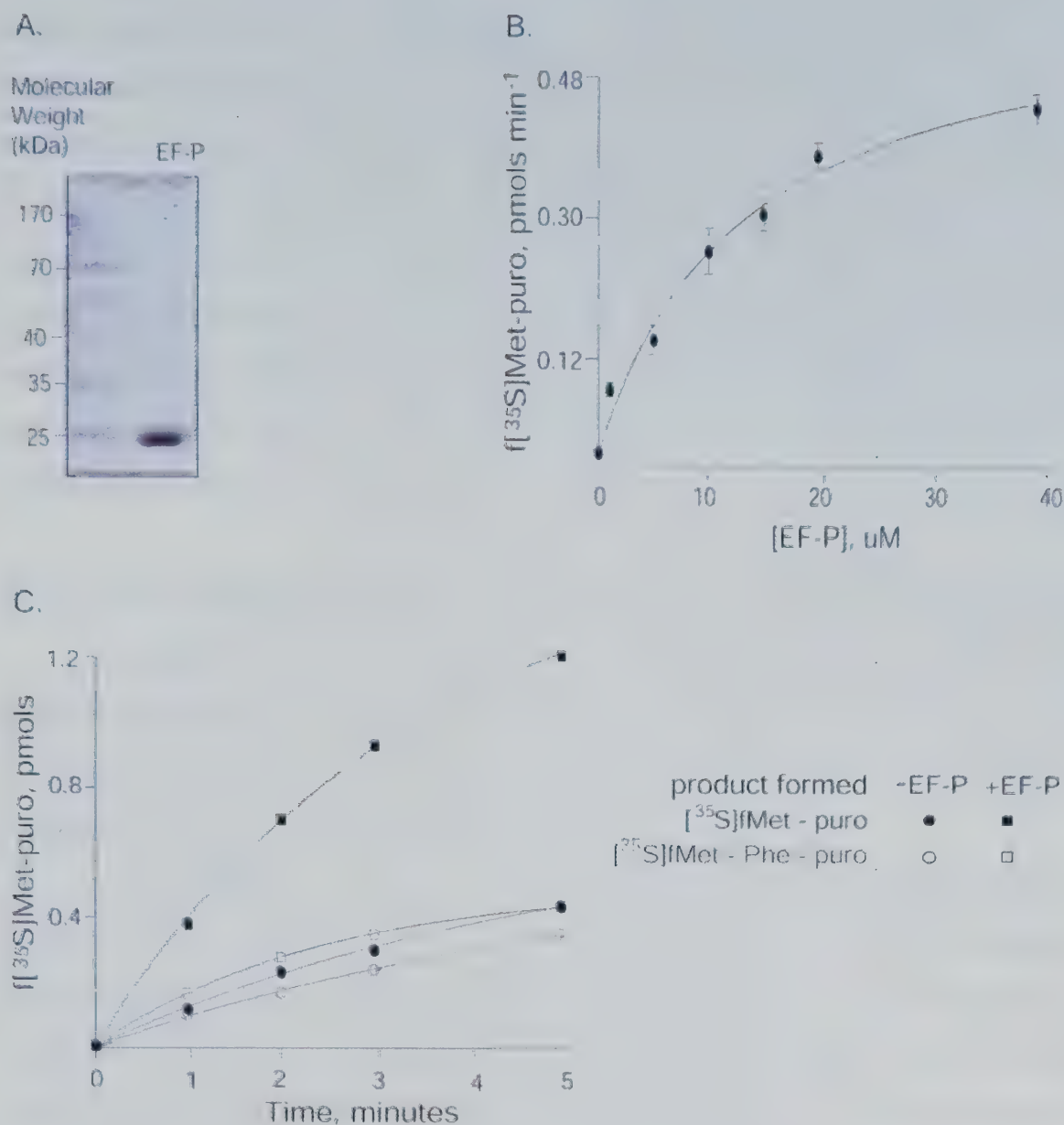


Fig. IV.5. EF-P stimulates peptide bond formation between puromycin and $f\text{Met-tRNA}^{f\text{Met}}$

A. 10 % SDS-PAGE analysis of the EF-P double purification procedure yield.

B. Initiation complexes containing $1\mu\text{M}$ 70S ribosomes, $1\mu\text{M}$ mRNA and $0.2\mu\text{M}$ $f[^{35}\text{S}]\text{Met-tRNA}^{f\text{Met}}$ were allowed to form for 30 minutes at 37°C and then examined upon addition of $1\mu\text{M}$ puromycin. Error bars represent the average of four independent experiments.

C. Initiation complex formation conditions are as described in panel B. $1\mu\text{M}$ Phe-tRNA^{Phe}, together with $1\mu\text{M}$ EF-G and $20\mu\text{M}$ GTP were incubated with the initiation complex for an additional 15 minutes at 37°C , where indicated. $1\mu\text{M}$ puromycin and $25\mu\text{M}$ EF-P were added, and the kinetics of the formation of $f[^{35}\text{S}]\text{Met-puromycin}$ (or $f[^{35}\text{S}]\text{Met-Phe-puromycin}$ respectively) were measured over five minutes.

However, from this experiment alone we cannot conclude whether the lesser stimulation of the Phe-puromycin product formation is due to the biochemical properties of the Phe-tRNA^{Phe}, as suggested previously [10], or to the fact that EF-P only stimulates the formation of the first peptide bond (in our case f[³⁵S]Met-Phe) [7, 15] and does not affect subsequent peptide bonds, e.g. Phe-puromycin. In order to fully understand whether EF-P functions solely during initiation by stimulating the formation of the first peptide bond, or whether it also acts sporadically during elongation by favoring the formation of certain peptidyltransferase reactions, one must investigate in detail the effects of EF-P with respect to initiation and elongation factors.

IV.2.3. IFs and EFs have no effect on EF-P-mediated peptide bond formation

In order to establish if EF-P acts as an IF [20], at a borderline step between initiation and elongation [2] or as an EF [43], we investigated EF-P's role in these two stages of the translational cycle by measuring the rates of f[³⁵S]Met-puromycin formation in presence of IFs and EFs.

First, we looked at the effects of the IFs over EF-P; we purified all three IFs as described in [44] to almost 99 % purity (Fig. IV.6., A.). We then examined the effects of the IFs on EF-P's stimulation of f[³⁵S]Met-puromycin formation. Initiation complexes were pre-formed as described above, and kinetics measurements were done in the presence and absence of EF-P. EF-P and each IF were in report of 1:1. As shown in Fig. IV.6., B., the addition of any IF to a preformed initiation complex did not notably stimulate the formation of f[³⁵S]Met-puromycin product and distinct peptidyltransferase stimulation effects were only observed upon concurrent addition of EF-P.

The fact that the IF's have little to no effect over EF-P's stimulation of peptide bond formation on pre-formed initiation complexes is not surprising, as the IFs are known to act only during initiation complex assembly, after which they dissociate from the ribosome [45].

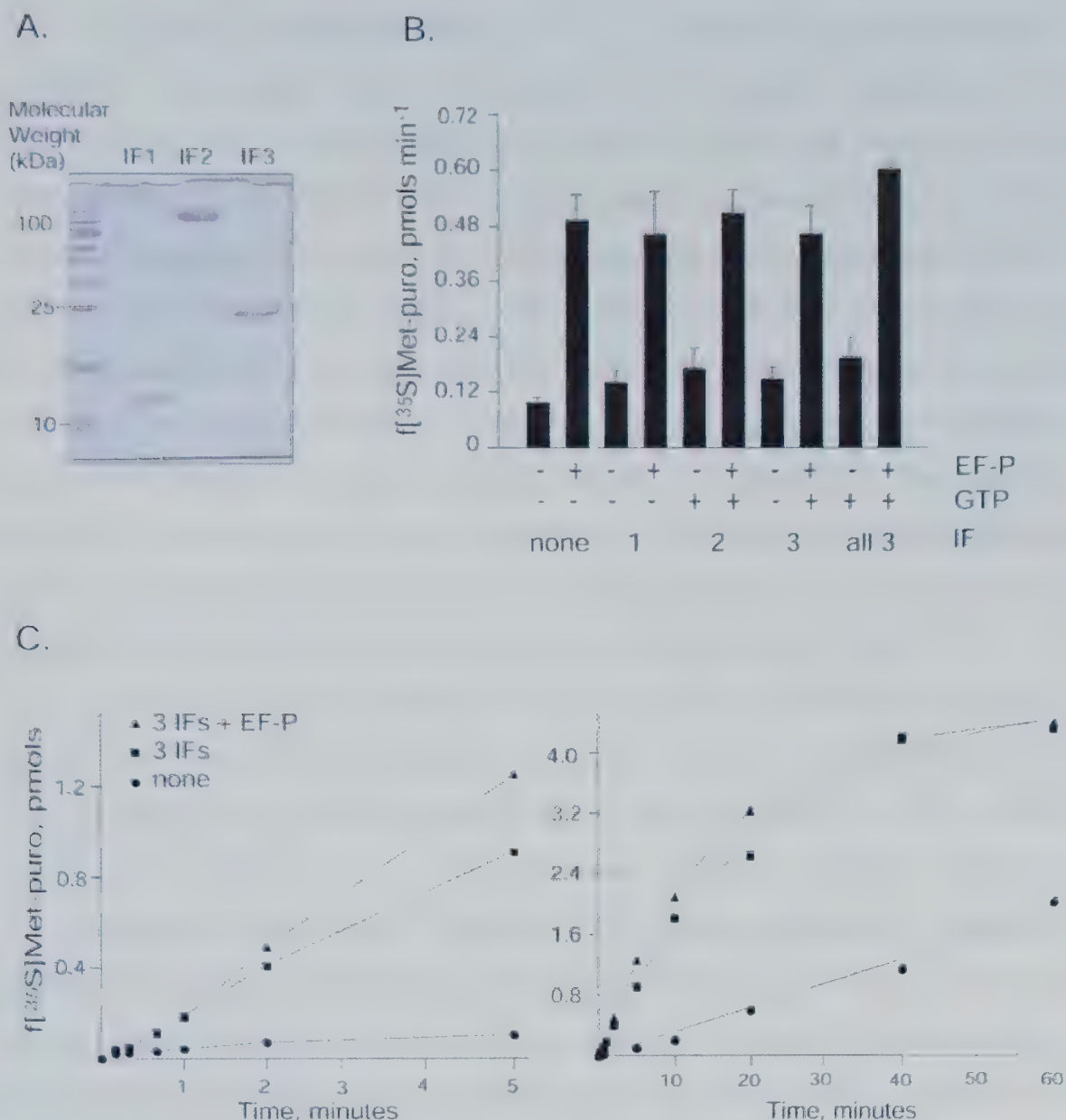


Fig. IV.6. Investigating EF-P's involvement in initiation

A. 16 % SDS-PAGE analysis of purified *E. coli* IFs.

B. 5 μ M IFs, 20 μ M GTP (where indicated), 5 μ M EF-P and 1 μ M puromycin were added to pre-formed initiation complexes (1 μ M 70S, 1 μ M mRNA, 0.2 μ M f[³⁵S]Met-tRNA^{fMet}). Error bars represent the average of three independent experiments.

C. 1 μ M 70S, 5 μ M IFs (where indicated), 20 μ M GTP and 1 μ M puromycin were rapidly mixed with 5 μ M EF-P (as indicated), 1 μ M mRNA and 0.2 μ M f[³⁵S]Met-tRNA^{fMet}. The reactions were allowed to proceed for 60 minutes at 37° C and the number of pmols of f[³⁵S]Met-puromycin formed was recorded as a function of time. The first graph is a shorter time frame (zoom-in) of the second, more extensive recording.

In order to investigate whether EF-P is involved in initiation complex assembly, we skipped the pre-assembly of ribosomal complexes. We concomitantly mixed 70S ribosomes, all three IFs, GTP and puromycin with EF-P, mRNA and f[³⁵S]Met-tRNA^{fMet}. By following the timely progression of the reactions containing IFs (Fig. IV.6., C), it appears that EF-P's stimulatory effect is not immediately manifested, which might indicate that it does not participate in the incipient phases of initiation complex formation. The observation that in the absence of pre-formed initiation complexes, the IF's had a marked stimulatory effect on f[³⁵S]Met-puromycin formation, which is comparable to that exhibited by EF-P, suggests that EF-P may be involved in stabilizing the complex after its partial or complete assembly onto the ribosome and therefore indirectly promote peptidyltransfer, which has been previously hypothesized by [12, 20].

Next, we turned our attention towards the EFs; we purified hexahistidine tagged EF-G and EF-Ts as described in [46], as well as EF-Tu, as described in [44]. All three EFs were approximately 95 % pure as shown by 10-12 % SDS-PAGE analysis (Fig. IV.7., A.). We pre-formed initiation complexes containing 70S ribosomes, mRNA and f[³⁵S]Met-tRNA^{fMet} and measured the kinetics of f[³⁵S]Met-puromycin formation in presence and absence of EF-P and EFs (EF-P and EFs were in report of 1:1), with GDP or GTP (as indicated). As shown in Fig. IV.7., B., EF-G, in the presence of either GTP or GDP, mildly inhibited EF-P dependent peptide bond formation rates. As EF-G has been reported to inhibit fMet-tRNA^{fMet} binding [47-48], this result is not surprising. Also, recent work shows that EF-G binding to the ribosome triggers a conformational change of the L1 stalk, and therefore allosterically affects the release of the deacylated tRNA from the E site [49]. Since the L1 stalk is observed by structural studies to interact with EF-P similarly to a tRNA [20], it is possible that the mild inhibition observed in the presence of EF-G is indirectly due to accelerated EF-P exit mechanisms. EF-Tu-GDP showed a similar effect as EF-G (Fig. IV.7., B.). EF-Tu is purified and stored in the GDP state [44], thus in the reaction entitled Tu/Ts in Fig. IV.7., B., 5 μ M EF-Ts and 20 μ M GTP were added together with puromycin and EF-P in order to catalyze the exchange of GDP for GTP on EF-Tu.

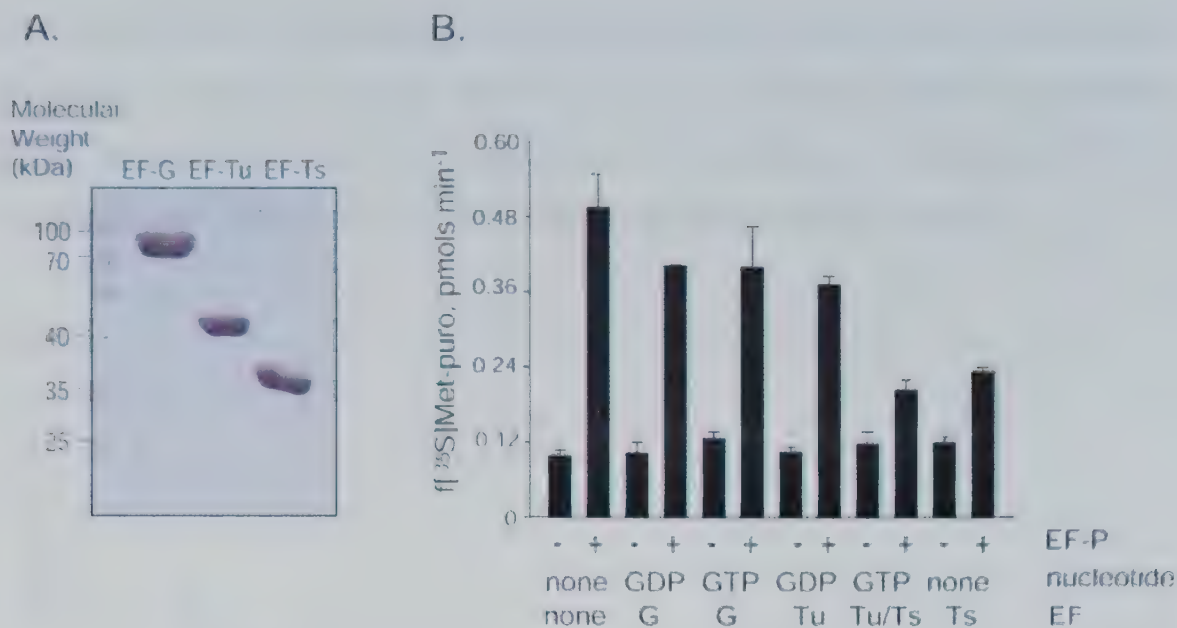


Fig. IV.7. EFs' effects on EF-P activated f[³⁵S]Met-puromycin formation

A. 10-12 % SDS-PAGE analysis of the final purification yield for all EF's.

B. 5 μ M each EF, 20 μ M GTP or GDP, 5 μ M EF-P (as indicated) and 1 μ M puromycin were added to preformed initiation complexes (1 μ M 70S, 1 μ M mRNA, 0.2 μ M f[³⁵S]Met-tRNA^{fMet}). Error bars represent the average of three independent experiments.

Interestingly, in the last two sets of reactions (Tu/Ts and Ts) in the presence of 5 μ M EF-Ts in both experiments, and regardless of the presence of EF-Tu, EF-P's ability to stimulate f[³⁵S]Met-puromycin formation was reduced significantly. We currently do not have a hypothesis explaining these effects of EF-Ts on peptidyl transfer stimulation by EF-P.

Recent studies on eIF-5A, the eukaryotic homolog of EF-P, hypothesize that both eIF-5A and EF-P may be in fact elongation factors which function in cooperation with EF-2 and EF-G respectively [32, 42-43]. We have shown that EF-G does not affect EF-P's ability to activate peptidyltransfer between f[³⁵S]Met-tRNA^{fMet} and puromycin (Fig. IV.7., B.). In order to fully evaluate any possible interactions between the two factors, we must now investigate EF-P's effect on EF-G mediated processes, GTP hydrolysis and translocation.

We investigated EF-G activity in presence and absence of EF-P under single round GTP hydrolysis and translocation conditions (Fig. IV.8.) [50]. The calculated reaction rates for EF-G mediated GTP hydrolysis and translocation

were very similar in the absence and presence of a 10 fold excess of EF-P over EF-G ($k_{GTP\text{ hydrolysis}} = 1.13\text{ s}^{-1}$ and $k_1, k_2\text{ translocation} = 0.22\text{ s}^{-1}, 0.01\text{ s}^{-1}$ respectively [50]), showing that EF-P does not affect EF-G's ability to hydrolyze GTP or translocate the mRNA and the tRNAs in the presence of the ribosome.

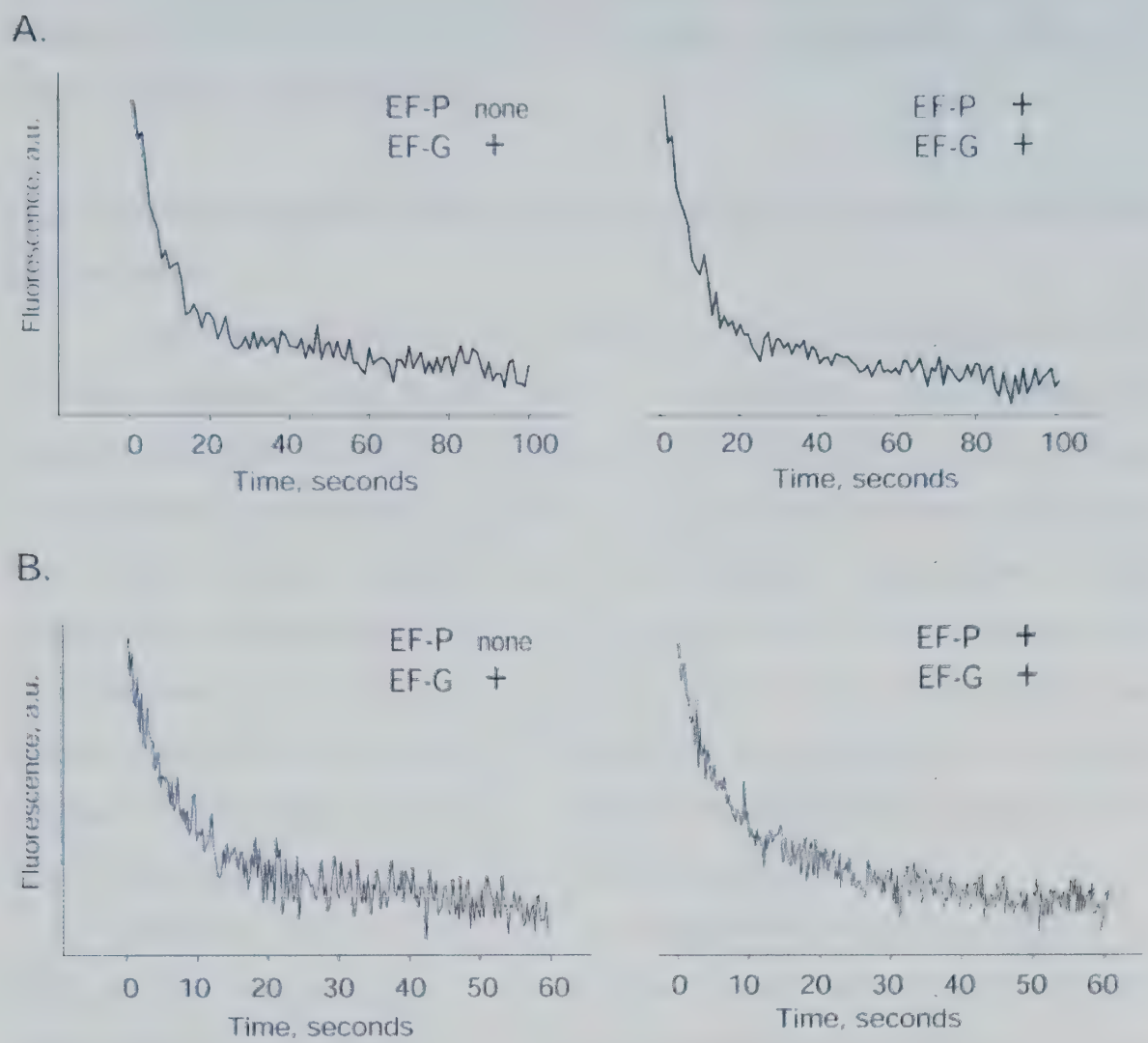


Fig. IV.8. EF-P effects on EF-G mediated single round GTP hydrolysis and translocation

A. Equal volumes of 10 μM mant-GTP and 10 μM EF-G, 24.4 μM 70S (with 100 μM EF-P as indicated) were rapidly mixed and the extent of mant-GTP hydrolysis was quantified by using a single exponential equation as in [50].

B. Equal volumes of preformed complexes of 0.4 μM pyrene labeled mRNA oligonucleotide, 0.5 μM 70S, 0.6 μM tRNA^{Met} (bound to the P site), 0.6 μM tRNA^{Phe} (bound to the A site), and 1 μM EF-G, 2 mM GTP (with 20 μM EF-P as indicated) were rapidly mixed and the extent of translocation was quantified using a double exponential equation as described in [50].

Taken together, our results suggest that *E. coli* EF-P and EF-G do not compete for ribosome binding. This is in agreement with structural studies placing EF-P in the P/E site, far from the EF-G binding site [20]. Although EF-G may allosterically influence E site tRNA release [49], its activity is exerted after the formation of the first peptide bond and therefore after EF-P has served its purpose, thus EF-G and EF-P are highly unlikely to catalytically affect each other's activities on the ribosome.

IV.2.4. Domains I and III of EF-P are important for the formation of the first peptide bond

Despite clear evidence that, unlike eIF-5A, EF-P is not involved in bacterial elongation with EF-G, these two proteins share strong structural similarities in terms of the first two domains [2]. The hypusine containing lysine has been shown to be located in domain I of eIF-5A [51-52] where it is part of a well conserved region, which in EF-P is ...K₃₁PGK₃₄G... (all residues in this chapter are *E. coli* numbers) [2]. The corresponding EF-P K34 is situated at the tip of domain I [2-3], and comes in contact with the CCA end of the P site initiator tRNA [20]. We mutated K34 and another conserved lysine at the tip of domain I, K31, to alanines, in order to evaluate the interactions between domain I of EF-P and the P site tRNA and rRNA (Fig. IV.9., B. and C.).

Domain II of EF-P, which has been suggested to mimic the elbow of a tRNA [3], has been shown to come into close contact with L1 [20], which is involved in E site tRNA release [21-24]. We decided to mutate residues Y76 and D91, situated at the elbow region of domain II to alanines, in order to evaluate EF-P interactions with L1 (Fig. IV.9., B. and C.).

Although absent in eIF-5a, domain III of EF-P contacts the anticodon end of the P site tRNA [20], thus it must play a role in the stabilization of the initiation complex. We therefore decided to create two functional mutants in domain III as well, 183A and 152A (Fig. IV.9., B. and C.). The purification yield of these mutants is approximately 99 % (Fig. IV.9., A.), similar to that of the wild type EF-P protein (Fig. IV. 5., A.).

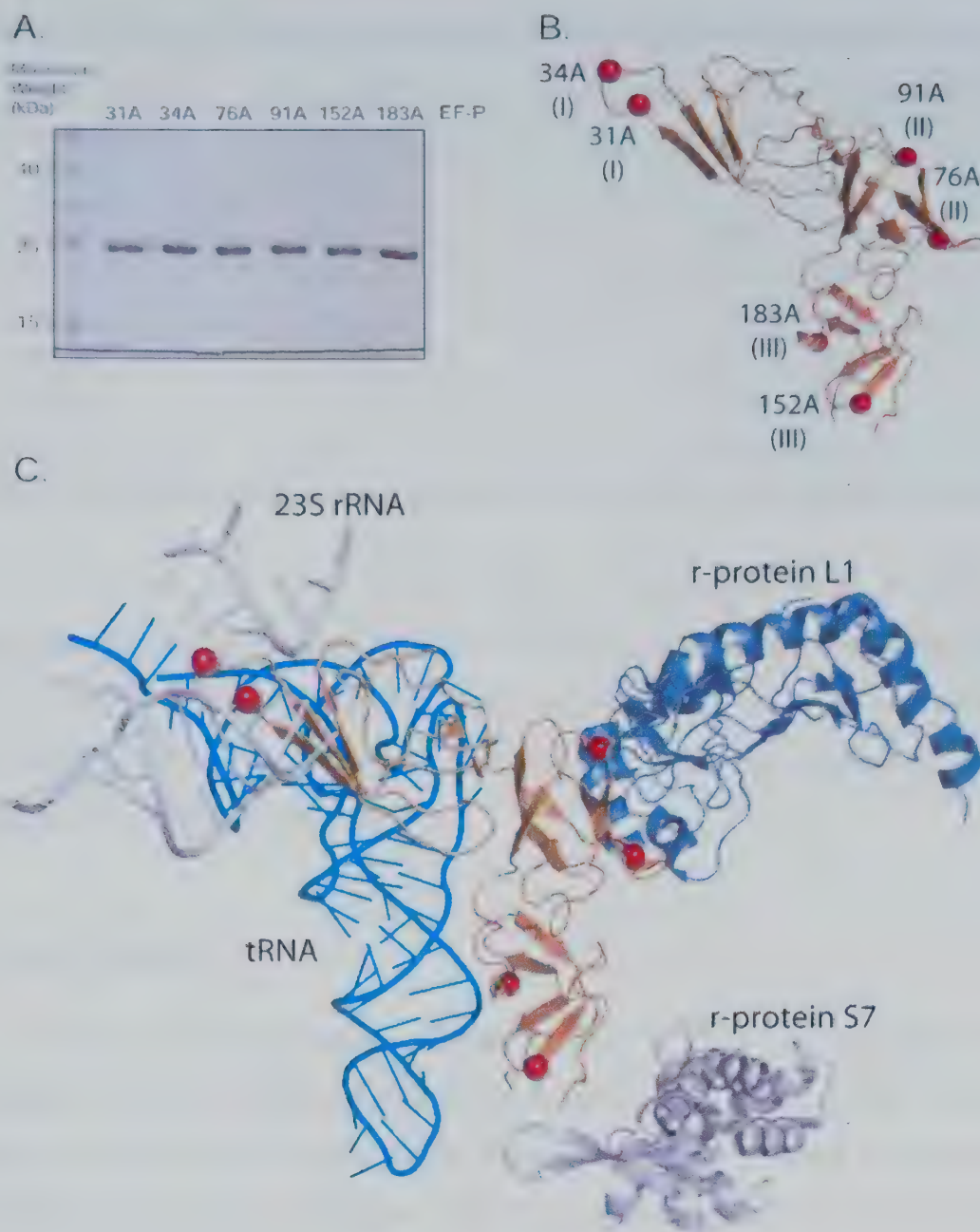


Fig. IV.9. Alanine mutants in EF-P and their interactions with the ribosome

A. 12 % SDS-PAGE analysis of the purification yield of the alanine mutants.

B., C. Pymol mapping of the position of the alanine mutants [20] with respect to the EF-P domains (B), and in the context of the ribosome (C) (PDB files 3HUW and 3HUX). EF-P is colored orange and the mutated residues are shown as red spheres, centered on the C_{α} atoms. For clarity, most of the rRNA is invisible, except for some P site 23S rRNA nucleotides, which are shown in light gray. tRNA is cyan and the r-proteins L1 and S7 are in teal and light blue, respectively.

We compared these mutants' fMet-puromycin peptide bond stimulation ability to that of the wild type protein, by employing the same experimental procedure described in Fig. IV.4., C.

Table IV.1. Effects of alanine mutations on EF-P' peptidyltransfer activity

EF-P *	K_M , μM	k_{cat} , pmols f[^{35}S]Met-puro min $^{-1}$
none	-	0.075 ± 0.012
WT	9 ± 1	0.54 ± 0.03
K31A	15 ± 1	0.54 ± 0.18
K34A	25 ± 4	0.39 ± 0.24
Y76A	14 ± 2	0.54 ± 0.09
E91A	13 ± 1	0.45 ± 0.03
Y152A	13 ± 1	0.42 ± 0.03
R183A	47 ± 1	0.54 ± 0.12

* Averages and standard deviations were calculated from at least three independent experiments.

As shown in Table IV.1., K34A is impaired both in terms of peptidyltransfer activation and in terms of binding to the ribosome, while K31A, the two domain II mutants and Y152A in domain III appear to be unaffected by the mutation. Y183A has the highest K_M value, indicating that residue 183 may be participating in ribosome binding.

IV.3. DISCUSSION

Ever since its functional characterization in 1975 [7], isolating EF-P's functional role in the translation cycle has proven to be an unexpected challenge. To date, it has not yet been clarified whether EF-P acts during initiation, at a borderline step between initiation and elongation or intermittently throughout elongation to facilitate the formation of certain peptide bonds.

Previous functional [6, 11-12] and structural studies [19] aiming to establish EF-P's binding site on the ribosome historically placed EF-P in the A, P or A/P sites. Recent crystallographic evidence shows EF-P in the P/E site, held in place by the r-protein L1 and making contact with the P site initiator tRNA, such as to stabilize fMet-tRNA^{fMet} in the P site and properly orient it in PTC [20]. The latter study contradicts previous hypothesis regarding EF-P's A, P or A/P site binding, but opens up interesting questions, such as how EF-P ends up trapped in between L1 and the fMet-tRNA^{fMet} in the first place, and how does it exit the ribosomal cavity after peptide bond formation.

With regard to the dynamics of EF-P binding to the ribosome there are several hypotheses worth considering: (1) EF-P binds to the ribosome together and cooperatively with the IFs and in doing so stabilizes initiation complex formation; (2) EF-P binds to the ribosome at the same time with, but independently from, the IFs; (3) EF-P comes into the ribosomal cavity through the E site after IFs' dissociation and/or ribosomal subunits assembly. Our experiments support the second or third hypothesis, as we show in this study that EF-P's ability to stimulate peptide bond formation is not enhanced by IFs, nor is EF-P able to immediately accelerate the peptidyltransferase reaction when initiation complexes are not pre-assembled (Fig. IV.5.).

In terms of primary and secondary structure, EF-P and its eukaryotic homolog, eIF-5A, share the first two domains [3]. However, despite the apparent structural mimicry, EF-P does not appear to share eIF-5A's involvement in translocation (Fig. IV.6.), nor does it functionally interact with EF-G, the bacterial homolog of EF-2 (Fig. IV.6. and IV.7.). In fact, previous antibiotic sensitivity studies also show that EF-P's peptidyltransferase activity is not impaired by antibiotics affecting translocation [6, 11].

In order to examine and interpret the role of specific residues of EF-P in the context of the ribosome, we have made extensive use of its ribosome bound crystal structure [20]. K34, which is the bacterial correspondent of the hypusinated lysine in eIF-5A [2] has not yet been clearly shown to be post-translationally modified in bacteria [11, 19], but has been proposed to be involved in the stabilization of the fMet-tRNA^{fMet} in the P site [20]. Indeed, by examining its position in molecular detail, K34 appears to lie in between, and directly on top of, 23S rRNA PTC (nucleotides 2253 to 2251) and the CCA end (nucleotides 74 to 76) of the initiator tRNA. K31 makes similar contacts, a few nucleotides lower than K34 (Fig. IV.10., A.). By taking into account the position of K31 and K34 with respect to the PTC and the 3' end of the tRNA, we can interpret our own functional mutagenesis experimental findings (Table IV.1.), i.e. that K34 (and, to a lesser extent, K31) are probably stabilizing the interaction between the initiator tRNA and the PTC during peptide bond formation [20]. On a related note, these

interactions may explain previous observations that the size of the aminoacyl moiety of the P site tRNA affects EF-P's peptidyltransferase activity [10] by steric hindrance effects between domain I of EF-P and bulky aminoacyl moieties in the PTC.

The mutated residues in domain II are shown in close contact with the r-protein L1 (Fig. IV.10., A. and B.), which may function to (1) “prop” EF-P in the P/E site and against the initiator tRNA and (2) to provide an exit mechanism for EF-P, as L1 has been shown to be involved in deacylated tRNA release from the ribosome [21-24]. Our mutagenesis experiments argue against the first hypothesis, as mutating these residues to alanines did not significantly affect the peptidyltransfer activity of EF-P (Table IV.1.).

Domain III residues appear to be in contact with both the anticodon loop region of the tRNA (Y152) and the r-protein S7 (R183) (Fig. IV.10., A. and B.). These two domain III residues appear to bridge the space between the base of the tRNA and S7, with Y152 contacting nucleotides 41 and 42 of the tRNA. Our mutagenesis experiments show that changing R183 to alanine renders the greatest difference in K_M value compared with the rest of the mutants, whereas mutating Y152 renders EF-P only mildly impaired in peptidyltransfer (Table IV.1.). In the context of the structural position of domain III and of the R183 residue, we conclude that EF-P contacts with S7 may be important for EF-P binding. On an additional note, protein S7 has been shown by crosslinking studies to be linked to the decoding site [53-54], which explains previous results of decoding site inhibitor antibiotics affecting EF-P's peptidyltransferase activity [6, 11].

Together, our functional mutagenesis results are interpretable in the context of recently released structural data [20]. Thus the tip of domain I overlays, but does not alter, the intimate contacts between the CCA end of the P site initiator tRNA and the PTC, with the residue situated at the very tip of domains I (K34) exerting the most intimate contacts with both the tRNA and the rRNA. Domain II contacts with exit protein L1 most likely do not affect EF-P stability in the P/E site and may only serve as a release mechanism for EF-P as soon as the first peptide bond is formed and the deacylated tRNA^{fMet} is pushed towards the E

site. Unlike domain II contacts with the ribosome, domain III contacts with r-protein S7 appear to be important for binding or stabilization of EF-P in the P/E site, as domain III appears held in place against the anticodon stem of the tRNA by S7 (Fig. IV.10., A. and B.). Interestingly, eIF-5A lacks domain III of EF-P [3], suggesting that its ribosome binding mechanism may differ from that of EF-P.

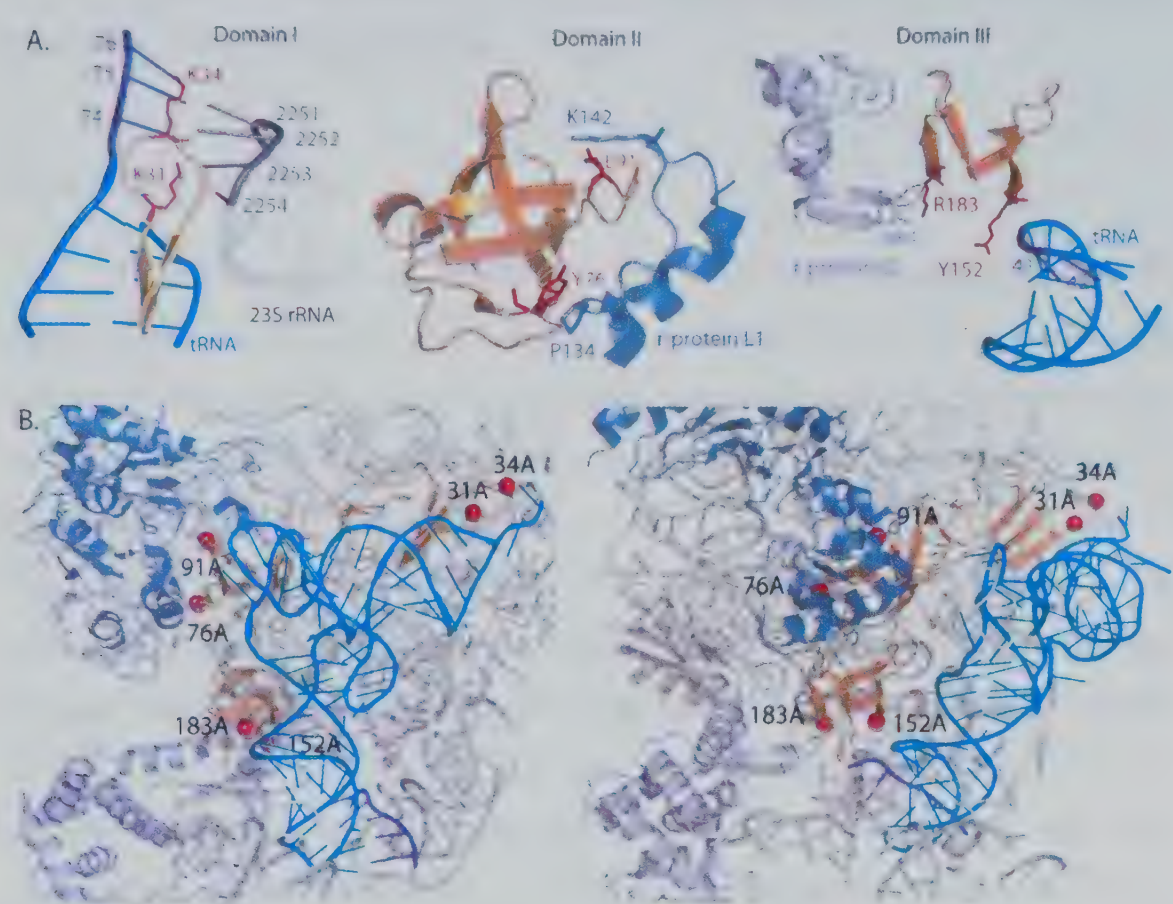


Fig. IV.10. The mutated residues in the context of the ribosome

The PDB files and the color code used are the same as in Fig. IV.9.

A. The mutated EF-P residues are shown in red sticks; for clarity, some of the compiling molecules are invisible. The tRNA and 23S rRNA nucleotides closest to the mutated residues are in dark blue and dark gray, respectively.

B. EF-P mutants (red spheres centered on the C α atoms) are shown in the context of the ribosome. rRNA is shown in light gray and the mRNA in dark purple.

Overall, our study suggests that EF-P is not directly involved in either initiation complex formation, EF-Tu GTP hydrolysis (data not shown) or EF-G mediated GTP hydrolysis or translocation. Also, EF-P domains I and III may functionally interact with the ribosome and the initiator tRNA so as to stabilize

EF-P against the P site tRNA, and in turn favor the formation of the first peptide bond by stabilizing the initiator tRNA in the 30S P site and in the 50S PTC [20].

It is left for further pursuit to examine whether EF-P also acts transiently throughout elongation, stabilizing other tRNAs in the P site and thus aiding in the formation of certain peptide bonds. Yet another question left to be clarified is how EF-P and the deacylated tRNA^{fMet} become released from the ribosome after the formation of the first peptide bond.

IV.4. CONCLUSIONS

This study examined EF-P's role in the bacterial translational cycle. We used a modified version of the initial puromycin based assay [7] to evaluate the influence of IFs and EFs over EF-P's ability to stimulate peptidyltransfer, as well as the importance for ribosome binding and peptide bond formation of conserved residues in EF-P.

EF-P's activity is unaffected by IF1, IF2, IF3 or a combination of the three, and it does not appear to affect initiation complex formation. We therefore suggest that EF-P does not participate in the initiation phase of protein synthesis. EF-P's eukaryotic homolog, eIF-5A, has recently been hypothesized to act in cooperation with EF-2, the eukaryotic homolog of EF-G [42-43]. Our study however, shows that EF-G does not affect EF-P's peptidyltransferase ability, nor does EF-P affect EF-G's ability to hydrolyze GTP or drive translocation on the ribosome.

Mutagenesis experiments suggest that domains I and III of EF-P are most important for peptidyltransfer, probably by a combination of ribosome binding and initiator tRNA stabilization in the P site. Since eIF-5A lacks domain III of EF-P [3], our data once again reiterate that structural mimicry does not translate into functional mimicry.

Overall, this study promotes the hypothesis that EF-P is neither an initiation nor an elongation factor, but rather acts at a transitional step between initiation and elongation, by favoring the formation of the first peptide bond.

IV.5. FUTURE DIRECTIONS

It has long been hypothesized that the ribosomal A and E sites allosterically influence each other such as to enhance A site decoding and binding mechanisms and to drive the movement of the tRNAs through the ribosomal cavity [55-56]. The r-protein L1 is known to shift conformations between “open” and “closed” states, based on ribosomal ratcheting and E site vacancy [21-24], but recent smFRET studies suggest that EF-G binding to the ribosome affects L1 conformational changes, and in turn promotes tRNA release from the E site [49]. Interestingly, L1 is also known to contact the elbow of the deacylated tRNA present in the E site [25-27] in a similar manner as it contacts domain II of EF-P [20], which in fact has been postulated to mimic the elbow of a tRNA [3].

The only crystal structure of EF-P bound to the ribosome available to date [20] shows the L1 stem pointing towards the P/E site and “trapping” EF-P adjacent to the initiator tRNA (see Fig. IV.9., C.). However, this study is lacking an A site aa-tRNA, which, if present, may alter EF-P’s contacts with L1 upon instant peptide bond formation and ribosomal ratcheting. In order to prevent EF-P release together with the deacylated tRNA^{fMet} through a possibly more “open” gate due to L1 conformational changes, one could use viomycin to trap a pre-translocational ribosomal complex containing mRNA, fMet-tRNA^{fMet}, aa-tRNA and EF-P. Viomycin is an antibiotic able to trap the ribosome in a EF-G·GDPNP-like ratcheted hybrid state, also known as pre-hydrolysis pre-translocation [57] (for details on viomycin, also see chapter III). Such a complex would provide a snapshot of the very next step in the EF-P cycle after the one shown in [10].

By comparing the existing structure of EF-P on the ribosome [20] with the above described complex, one can glean great insight into EF-P interactions with the tRNA^{fMet} in the absence of the fMet- moiety, as well as the in the orientation of EF-P with respect to the ribosomal subunits as the deacylated tRNA^{fMet} has moved from the P/P to the P/E position. The interactions between EF-P and the r-protein L1 would also be interesting to explore. As EF-P may be pushed by the initiator tRNA closer to the E site, L1 would most likely maintain its connection to domain II of EF-P, and in turn guide the factor out of the ribosomal cavity, in a

tRNA-like release mechanism. With this approach we would gain insight not only into EF-P release mechanisms, but also into L1 conformational changes associated with ribosomal ratcheting.

IV.6. MATERIALS AND METHODS

IV.6.1. Reagents and buffers

Some of the reagents delineated in chapters II.6.1. and III.6.1., purchased from the same sources, were also used for the experiments described in this chapter. In addition, phenylalanine, methionine and folinic acid were purchased from Sigma Aldrich Ltd. [³⁵S]-methionine (500 µCi) was purchased from Perkin Elmer.

E. coli ribosomes and gene 32 mRNA were prepared exactly as described in chapter II.6.

Buffers used exactly as in chapters II and III are numbered 1 to 23, buffers used in this chapter are numbered from 24 to 28. All experiments conducted in this chapter were done in buffer 24. **Buffer 24:** 30 mM HEPES pH 7.7, 15 mM MgCl₂, 50 mM KCl and 1 mM DTT; **buffer 25:** 20 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1 mM 2-mercaptoethanol and 5 % glycerol; **buffer 26:** 10 mM Tris-HCl pH 7.5, 30 mM NH₄Cl, 10 mM MgCl₂ and 6 mM β-mercaptoethanol; **buffer 27:** 500 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 500 mM KCl, 50 mM DTT and 1 mM EDTA; **buffer 28:** 200 µg/ml BSA, 1 M NaOAc pH 5.2, 2.5 % (v/v) trichloroacetic acid and 50 % (v/v) ethanol.

IV.6.2 EF-P cloning, mutagenesis, expression and purification

E. coli cloning was done by Kevin Wilson. The gene encoding EF-P was amplified from *E. coli* MRE600 genomic DNA by PCR using the following primers: 5'-GCG CGG CAG CCA TAT GGC AAC GTA CTA TAG CAA CG-3' and 5'-GCG CGG CAG CCT CGA GCT TCA CGC GAG AGA CGT ATT C-3'. The resulting DNA product was cut with restriction enzymes *Nde*I and *Xho*I (underlined sequences) and inserted between the corresponding sites in pET24b+

(Novagen). The EF-P coding DNA sequence in the resulting expression plasmid was as previously reported [58], except that it encoded a C-terminal hexahistidine His tag (Glu-Leu-His₆) and that one silent mutation was noted at codon 167 (TTT → TTC). The expression plasmid was transformed into *E. coli* BL21(DE3). The resulting expression strain and plasmid were stored separately at - 80° C.

The following primers were created: **K31A**: 5'-GCC TGG CCT TTA CCC GGA GCT ACG AAT TCA CTC GC-3', **K34A**: 5'-GCG AGC AAA TGC CTG GCC AGC ACC CGG TTT TAC GAA TTC ACT CGC-3', **Y76A**: 5'-CTC ACC GTC GTT AGC CAG GTA AGT CAG-3', **E91A**: 5'-GCA GAC AGC TGA GCG AAA GTT TC-3', **Y152A**: 5'-CAG GGT AGC CGG AGC GCC ACC AGT ACC-3', **Y183A**: 5'-CGC GAG AGA CAG CTT CAC CAG AGC-3'.

The mutagenesis, expression and purification of EF-P was done exactly as described for EF-G in chapter II, except for the second (size exclusion) protein purification step, which was replaced by anion exchange column purification.

The dialysis product from the Ni-NTA column was loaded onto a Resource-Q anion exchange chromatography column, equilibrated in buffer 25 with 0.2 M KCl and connected to an ÄKTA purifier apparatus (Amersham Biosciences). The column was washed with a linear gradient of KCl (0.2 M to 1 M) in buffer 25. EF-P eluted at approximately 400 mM KCl. The peak profile was analyzed using the Unicorn software and showed only minimal contamination (less than 1 %) of other protein products. The elution fractions were analyzed by 12 % SDS-PAGE and the purest peak fractions were pooled together, dialyzed overnight at 4° C against buffer 7 and stored at - 20° C until further use. Protein concentrations were measured by the Bradford assay (using BSA as a standard).

IV.6.3. Purification of other translation factors

Plasmids expressing IF1, IF2, and IF3 were generously provided by A. La Teana and C. Gualerzi. IF1, IF2, and IF3 were purified by Duane Gingras as described in [44].

Plasmids expressing hexahistidine tagged EF-Tu and EF-Ts were generously provided by B. Kraal. EF-Tu and EF-Ts were purified using the protocol described in [59] as a guideline, with the following modifications: EF-G, EF-Tu and EF-Ts were purified by Ni-NTA only, exactly as described for EF-G in chapter II.6. In the case of EF-Tu, all buffers used had the same composition as described in chapter II.6., except 10 mM GDP was added.

IV.6.4. *E. coli* S100 extract preparation

20 g of MRE600 cells were lysed in buffer 26 by using a French Press (at least two pulses of 18,000 p.s.i). The S30 extract was obtained by centrifugation of the crude extract solution (30,000 x g, for 30 minutes at 4° C). The crude S100 extract was obtained by centrifugation of the S30 extract (100,000 x g for 120 minutes at 4° C). In parallel, 12 g of dry DEAE-cellulose was mixed with 100 ml of ice cold buffer 26 by slow stirring in a beaker for 15 minutes. The slurry was then let to settle at the bottom of the beaker and the supernatant was decanted. The washing of the slurry was repeated three more times.

The crude S100 extract was added to the slurry and mixed gently by stirring. The solution was then transferred to a Buchner funnel lined with GF/A Whatman filter paper and filtered under vacuum into a side-arm Erlenmeyer flask. The DEAE-cellulose was thoroughly washed with 1 L of ice cold buffer 26, and the absorbance of the effluent was monitored by spectrophotometry. The washing was repeated until the $A_{280\text{nm}}$ was less than 0.01.

The DEAE-cellulose was then packed into a column at 4° C and the bound aminoacyl tRNA synthetases were then eluted with a solution of 0.25 M NH_4Cl in buffer 26. Under these conditions, nucleic acids, ribosomes and basic proteins will remain bound to the DEAE-cellulose resin. 2.5 ml fractions were collected and the synthetase containing fractions (yellow) were pooled together, combined with 10 % (v/v) glycerol, and 10 μl aliquots were quick-frozen in liquid nitrogen and stored at - 80° C until further use.

The total protein concentration was calculated as $DF \times (A_{280nm} - A_{234nm})/3$, where DF represents the dilution factor. The yield was calculated as (total protein concentration $\times$ total volume)/20 g of MRE600 cells.

IV.6.5. f[35 S]Met-tRNA^{fMet} and Phe-tRNA^{Phe} preparation

Folinic acid (Sigma) was stored in 41 mM β -mercaptoethanol and 180 mM HCl. Previous to use, folinic acid was neutralized to pH 7.0 by titration of 0.01 M KOH solution. 2.4 μ M tRNA^{fMet}, 10 μ M methionine (with 0.086 μ M [35 S]-methionine), 175 μ M folinic acid-KOH pH 7.0, and 2 mM ATP were incubated for 30 minutes at 37° C in presence of S100 extract in buffer 27. The Phe-tRNA^{Phe} was charged in the exact same manner as the f[35 S]Met-tRNA^{fMet}, except 10 μ M unlabelled phenylalanine were used to charge commercial (Sigma) yeast tRNA^{Phe} by the *E. coli* aminoacyl tRNA synthetases found in the S100 extract.

Charged tRNAs were extracted in presence of 1/10 (v/v) 3M NaOAc pH 5.2 with phenol pH 6.6 and chloroform (ratio 1:1, v/v), followed by overnight precipitation at - 20° C in presence of 3X (v/v) 100 % ethanol. The precipitated charged tRNAs were harvested by centrifugation (10 minutes at 13,800 x g), washed with 80 % (v/v) ethanol and stored at - 80° C in 30 mM NaOAc pH 5.2.

Charging efficiency and final concentration of f[35 S]Met-tRNA^{fMet} was determined to be over 90 % based on counting the radioactivity of [35 S] (cpm, counts per minute) after filtration under vacuum in buffer 28 through GF/C Whatman filters, which retain tRNA, but not free [35 S]-methionine. The concentration of Phe-tRNA^{Phe} was determined by spectrophotometry by using an extinction coefficient of tRNA $\epsilon_{280} = 25$ g/l.

IV.6.6. Peptide bond formation assays

All reactions were conducted in a 20 μ l final volume. Initiation complexes containing 1 μ M *E. coli* 70S ribosomes, 1 μ M mRNA, and 0.2 μ M f[35 S]Met-tRNA^{fMet} were formed for 30 minutes at 37° C.

In some experiments (Fig. IV.4.), after initiation complex formation as described above, 1 μ M Phe-tRNA^{Phe} was bound to the A site by incubating the

reaction at 37° C for 15 minutes. 1 μ M EF-G with 20 μ M GTP was used to translocate (37° C for 15 minutes) deacylated tRNA^{fMet} and f[³⁵S]Met-Phe-tRNA^{Phe} to the E and P site respectively. Initiation and elongation factors (Fig. IV.5. and IV.6.), when present, were 5 μ M, while nucleotides (GTP or GDP) when present, were 20 μ M.

Puromycin (1 μ M) was added simultaneously with 25 μ M (Fig. IV.4.) or 5 μ M (Fig. IV.5. and IV.6.) EF-P. In Fig. IV.5., C. only, initiation complexes were not pre-assembled prior to EF-P/puromycin addition, and 10 μ l of 2 μ M 70S, 10 μ M IFs, 40 μ M GTP and 2 μ M puromycin were combined with 10 μ l of 10 μ M EF-P (where indicated), 2 μ M mRNA and 0.4 μ M f[³⁵S]Met-tRNA^{fMet}.

Following the mixing of equal volumes of reaction components, as described above (defined as $t = 0$), reactions were incubated at 37° C and aliquots (4 μ l) were removed after various time intervals (as indicated). The 4 μ l aliquots were immediately quenched with 500 μ l 1 M NaOH, which hydrolyzes the ester bond of un-reacted f[³⁵S]Met-tRNA^{fMet}. From each 504 μ l quenched sample, a 100 μ l aliquot (sample A) was removed and diluted into scintillation fluid (5 ml). 1 ml ethyl acetate was added to the remaining 404 μ l sample, mixed vigorously (2 minutes) by vortex, and centrifuged (13,600 x g, 1 minute) to separate the liquid phases. An aliquot (800 μ l, sample B) of the aqueous ethyl acetate phase (containing the extracted f[³⁵S]Met-puromycin product) was removed and diluted into scintillation fluid (5 ml). The radioactivity (cpm) of samples A and B was counted using a LS 6500 liquid scintillation counter.

An example of primary data in terms of EF-P effects (pmols of f[³⁵S]Met-puromycin formed over time) is shown in Fig. IV.5., C., in dark squares. The EF-P concentration is 25 μ M.

Initial reaction velocities (v_o) were calculated as pmols of f[³⁵S]Met-puromycin s⁻¹ formed. Plots of v_o as a function of EF-P concentration were fitted to the Michaelis-Menton equation: $v_o = V_{max} \times [EF-P] / ([EF-P] + K_M)$ by using Sigmaplot 2001 software.

IV.6.7. EF-G single round mant-GTP hydrolysis and translocation assays

Fluorescence experiments were performed using a PTI QM-6 (Photon Technology International) equipped with a LPS-220B lamp and with a stopped-flow MiniMixer (KinTek Corporation) device, which rapidly mixes two separate samples of approximately 180 μ l each.

Single round mant-GTP hydrolysis assays were performed exactly as in [50], except in buffer 24. Equal volumes of 10 μ M mant-GTP and 10 μ M EF-G, 24.4 μ M 70S (with 100 μ M EF-P as indicated) were rapidly mixed and the fluorescence drop was recorded. The extent of GTP hydrolysis was quantified using Sigmaplot 11 software and was fitted to the equation: $Ft = F_{\infty} + \Delta F1 \times \exp(-k_{obs} \times t)$.

Single round pyrene-mRNA translocation was done exactly as described in [50] and in chapter II, with the following minor modifications: (1) the reactions were carried out in buffer 24; (2) the final EF-G concentration was 0.5 μ M, and (3) where indicated, EF-P was added to a final concentration of 10 μ M. The double exponential equation fitted to the data was: $Ft = F_{\infty} + \Delta F1 \times \exp(-k_1 \times t) + \Delta F2 \times \exp(-k_2 \times t)$.

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V. BACTERIAL SYSTEMS ARE VALUABLE TOOLS FOR STUDYING THE MECHANISMS OF TRANSLATION AND THE PATHOGENEITY OF ANTIBIOTIC RESISTANCE

V.1. *E. coli* SYSTEMS

Escherichia coli (or *E. coli*), discovered in 1885 by German pediatrician and bacteriologist Theodor Escherich, is a Gram negative, facultative anaerobic, rod shaped bacterium. Although some *E. coli* strains can cause serious infections in humans, most are harmless and live in the gastrointestinal tract of endotherms in symbiosis with their hosts, by producing vitamin K or by preventing the establishment of other pathogenic bacteria within the intestine.

E. coli can be grown easily in the laboratory and its genetics are comparatively simple and easily manipulated or duplicated through metagenic processes, making it one of the best studied prokaryotic model organisms, and an important model species in biochemistry. *E.coli* also proved to be an ideal biotechnological tool for studying the adaptation of synthetic bacterial ecosystems to complex habitat landscapes generated at the nanoscale.

Moreover, the relevance of studying *E. coli* systems also spans microbiology and medicine, as *E. coli* often carry multidrug resistant plasmids and are a frequent member of natural biofilms, where many species of bacteria exist in close proximity with each other. Under these proximity conditions, *E. coli* participates in plasmid exchange with other species, including *S. aureus*. Such properties of *E. coli* are particularly relevant in the epidemiological context of the emergence of multidrug resistant bacterial strains, such as methicillin resistant *S. aureus* (MRSA), especially since it has been reported that the rate of adaptive mutations in *E. coli* is on the order of 10^{-5} per genome per generation, which is almost 1,000 times higher than previously estimated [1].

We chose this highly representative, yet simple and adaptable, bacterial system, to study two of the most conserved and intriguing translational protein factors: (1) EF-G, in terms of ribosome activated GTP hydrolysis and of fusidic acid resistance; and (2) EF-P, whose eukaryotic homolog has been proposed to function alongside EF-G's eukaryotic correspondent, eEF-2.

V.2. EF-G AND EF-P AS TRANSLATIONAL PROTEINS

Although discovered no more than 10 years apart, there is an almost 30 years gap in the knowledge we have on these two essential translational factors.

EF-G has been extensively studied, both functionally and structurally, which led to great advances regarding the mechanism of translocation. EF-P research, on the other hand, is at a much earlier stage, and although recent structural data visualized its binding site onto the ribosome [2], little is known about how EF-P actually functions in translation.

In this chapter, I will discuss the relevance of our findings regarding EF-G and EF-P in the broader context of bacterial translational research.

V.2.1. The role of the switch regions of EF-G in GTP hydrolysis

EF-Tu and EF-G are ribosome activated GTPases which control tRNA delivery and translocation, respectively. The mechanism of GTP hydrolysis activation in EF-G and EF-Tu has long been an intriguing step of the elongation process. Although both proteins are able to intrinsically hydrolyze GTP in solution, their GTP hydrolysis activity is greatly accelerated in the presence of the ribosome, which is believed to promote the necessary conformational changes within the GTPase domains of the two EFs. The ribosomal GAC has been proposed to use r-protein L7/L12 to recruit the EF's to the ribosome [3], distinguish between EF-Tu and EF-G [4], and probably also activate GTP hydrolysis [4-6].

In EF-G, switch I, switch II and domain III residues situated at the interface between domains I and III, have been shown to create a hydrophobic pocket (Fig. I.6.), which may protect the GTP molecule from water attack in the (free)GTP state of EF-G (see Fig. III.1.) [7]. This hydrophobic pocket may undergo progressive minimal conformational rearrangements which cooperatively promote water access to the γ phosphate of GTP during L7/L12 recruitment, ribosome binding and/or recognition and GAC activation. Once GTP hydrolysis is activated on the ribosome, the G domain may undergo more significant conformational changes in terms of the two switch regions and inter-domain

contacts, which cascade to the other EF-G domains and eventually lead to the extended shape of EF-G [8] necessary for translocation [9], as well as to GDP release and EF-G recycling [10].

In order to gain more insight into these mechanisms, we focused on the components of the hydrophobic pocket protecting the nucleotide in the GTP state: switch I, switch II and domain III. The switch regions are postulated to unlock the function of GTPases upon GTP hydrolysis, while in EF-G, domain III deletion has been shown to abolish GTP hydrolysis activation by the ribosome and stimulate EF-G's intrinsic GTP hydrolysis activity in solution [11]. Domain III forms the interface onto which domains III, IV and V rotate and extend for translocation [8], and although it does not come in direct contact with the GAC, it contacts both ribosomal subunits, and may serve as an EF-G "recognition" mechanism of the ribosome's conformational state [12].

Our mutagenesis studies (chapter II) suggest that by disrupting the interactions between switch II and domain III, one creates a gap in the hydrophobic pocket and in the inter-domain interface contacts. It is also possible that other intrinsic domain I and/or domain III contacts have also been disrupted by these mutations, as it has been shown that single residue mutations in this region of EF-G lead to extensive rearrangements of the G domain in general, and of the switch regions in particular [13-15]. Overall, our mutants may have created an alternative path for nucleotide attack by water molecules in solution, which could explain the uninhibited ability of some mutants to hydrolyze GTP in the absence of the ribosome. Whether these mutagenesis dependent conformational rearrangements, if any, in fact mimic the "GTPase active" form of EF-G on the ribosome, remains for future structural studies to determine.

Another intriguing implication of our switch II/domain III study lies with the ability of the mutants to drive the progression of the EF-G cycle on the ribosome. Although able to bind to the ribosome, many of the alanine mutants are defective in activating GTP hydrolysis on the ribosome. These findings could be explained as follows: (1) that domain III interactions with the ribosome are indeed required for GTP activation [12], and/or (2) the interactions between switch II and

domain III are needed to maintain the integrity of the GTPase center of EF-G. However, while both switch II and domain III residues' mutation affect ribosome activated GTP hydrolysis of EF-G, the mutant situated at the tip of switch II (F95A) is by far the most severely impaired in translocation, suggesting that switch II may function as a pivot at the inter-domain interface, and by a “ball and socket” type of mechanism, stabilize the partial rotation and extension of the EF-G molecule, which unlocks progression from GTP hydrolysis into translocation. This idea is supported by structural studies showing that switch II maintains a relatively stable position (pointing towards domain III) throughout GTP hydrolysis [7] and translocation [12].

With regard to the third component of the hydrophobic pocket, switch I, all crystal structures of EF-G·GDP to date [12-17] show a disordered switch I region. Our study is the first to map the movement of the switch I loop of EF-G with respect to the ribosome, between the GTP and GDP states of EF-G. We used a combination of functional, trypsin digestion, and structural, hydroxyl radical probing, techniques to show that following GTP hydrolysis and 30S translocation, switch I flips outward from its “tucked in” GTP conformation [7], becoming exposed, unanchored, and dynamically flipping outside of the ribosomal cavity. In light of our switch II/domain III work, switch I flipping outward may not only facilitate nucleotide release and EF-G recycling [10], but it may also promote the extension of the EF-G molecule needed for translocation, by loosening the interactions between domains I and III. Although this conformational change of switch I is not unique among GTPases [18], it is opposite from that previously observed by crystallographic studies of EF-Tu in solution [19-20], and similar to that observed by very recent structural studies of ribosome bound EF-Tu [21-23]. Thus we are left with the open question of whether switch I undergoes similar movements in the GDP state of EF-G and EF-Tu after all.

Additionally, recent studies of Ras proteins have envisioned a role for switch I in activation of intrinsic GTP hydrolysis [24], as well as a role for switch II in mediating GTP hydrolysis and nucleotide exchange as well as in sensing the on and off states [25].

V.2.2. The role of the switch regions of EF-G in fusidic acid resistance

Our study of the physiology of the EF-G switch regions enriches our understanding of conformational rearrangements of the inter-domain interface which preclude and condition fusidic acid binding, and in turn, fusidic acid resistance and sensitivity mechanisms.

As switch I opens and the EF-G molecule extends for translocation the binding site for the antibiotic becomes accessible [10, 12], which explains fusidic acid sensitivity.

Our switch II work centers on residues which have been found to harbor spontaneously arisen fusidic acid mutations in *S. typhimurium* and *S. aureus* EF-G, and which map at the interface between domains I and III [15], where fusidic acid binds [12] (Fig. V.1.).

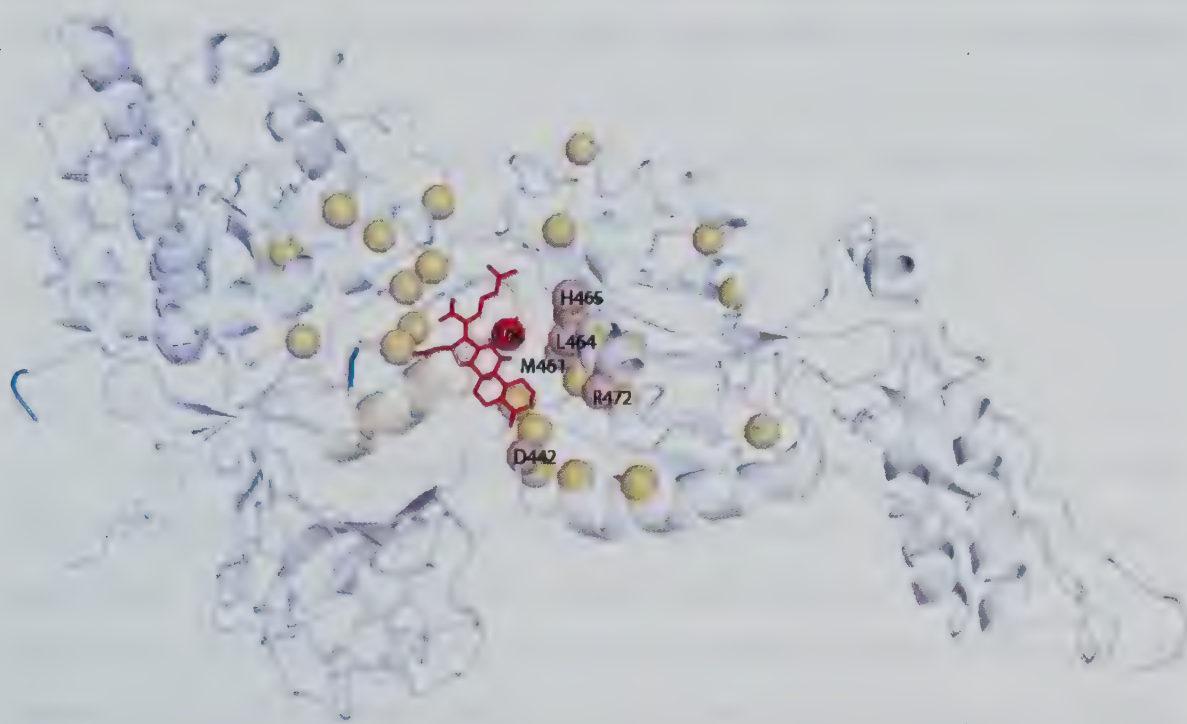


Fig. V.1. Spontaneous fusidic acid resistant mutants in EF-G

T. thermophilus EF-G (PDB 1WRI) is shown in white; switch I is disordered and colored blue and switch II is colored in light orange. EF-G residues harboring fusidic acid resistant mutants are shown as spheres centered on the Ca atoms, mostly colored in orange. F95 and domain III residues (D442, M461, L464, H465 and R472) are highlighted in red and salmon, respectively. Fusidic acid is shown as red sticks. All residues are *E. coli* numbering.

F95 (red), the residue situated at the tip of switch II has been shown to host the strongest fusidic acid resistant mutant known to date (F95L), while some of the residues we mutated in domain III (salmon) have also been shown to harbor fusidic acid resistant mutations. Also, compared with the wild type protein, all alanine mutants exhibited 10 to 300 fold increased resistance to fusidic acid, suggesting that, unlike switch I, switch II and domain III residues play an important role in fusidic acid resistance.

Still, more than one antibiotic resistance mechanism is often employed for a given bacterial target. Spontaneous mutations in EF-G conferring fusidic acid resistance have also been identified within the G domain, as well as in domain V (Fig. V.1.), suggesting that these mutations may target key structural elements involved in conformational changes needed for EF-G inter-domain interface opening and/or EF-G release [15]. Thus, studying fusidic acid resistance mechanisms may advance research into the physiology of the translocational machinery.

In conclusion, by studying in parallel the physiology and the pathophysiology of the ribosomal machinery, one can cooperatively advance the specialized knowledge of both processes.

V.2.3. EF-P

Despite almost 30 years of research, little is known today about the details of the translational role of this small protein factor. Many of the initial hypotheses regarding EF-P's function have been shown to be incorrect (i.e. EF-P's binding site on the ribosome), however it is still accepted that EF-P promotes the formation of the first peptide bond [2, 26] and that its tRNA-like structural shape enables EF-P to bind to the ribosome and make ribosomal contacts similar to a tRNA [2, 27].

Another “structural mimicry” hypothesis correlates EF-P and its eukaryotic structural correspondent, eIF-5A [27]. It has been suggested that, like eIF-5A and eEF-2, EF-P and EF-G would function in cooperation during elongation [28-30], however the existing literature on both EF-G and EF-P

support no such functional relationship. Also, unlike eIF-5A, which has been shown to function as a GAP for eIF-2 [30], EF-P has not been shown to be functionally related with bacterial IF-2. Moreover, domain III of EF-P, which is not present in eIF-5A [27], may play an important role in initiator tRNA stabilization in the P site [2] as well as in ribosome binding (chapter IV).

In conclusion, unlike in the case of the tRNA mimicry hypothesis, there does not seem to be any experimental support for functional mimicry between EF-P and eIF-5A, once again reiterating that structure and function do not necessarily condition one another.

V.3. THE CONNECTION BETWEEN BACTERIAL SYSTEMS AND PRACTICAL APPLICATIONS

The goal of fundamental, or basic, research is to gain knowledge and understanding of the physical world, without regard to whether or not it will be of any immediate commercial or practical use. Fundamental research arises out of curiosity, often combines seemingly unrelated facts, and explores unknown fields necessary to make new discoveries. This is different from applied research, in which scientific investigation aims to discover a solution to a given practical problem. Applied research however depends on basic research, as practical problems are often solved by insight into the most fundamental characteristics of nature, which prompts innovations, new developments and solutions to old problems.

As an illustration, while multidrug resistant bacterial strains are emerging and antibiotic therapy is running out of options, many of the alternative approaches to antibiotherapy come from fundamental research conducted in bacterial systems, such as RNAP inhibitors (myxopyronins) or phage therapy, an approach that has been shown to be efficient against some pathogenic bacteria, including *E. coli* and *S. aureus*. Other solutions can be found by purifying demonstrated bioactive substances extracted from plants (phytochemicals) or from Archaea (archaeocins).

Although autonomic basic research is essential for nourishing the expansion of future scientific knowledge, in the latter decades, the border between basic and applied research is slowly but surely fading, as the independent study of all branches of science is giving way to cooperative, multidirectional and multidisciplinary research. As illustrated by our own scientific interests, bacterial translational apparatus study spans biochemistry, microbiology and medicine, linking detailed structural and functional knowledge of bacterial elongation with antibiotic resistance and sensitivity mechanisms. In the end, such cooperative study provides the key tools for effective antibacterial drug design, thus contributing to limiting the spread of antibiotic resistant infections.

For now, the merging between basic and applied bioscience gains more and more acceptance in the scientific world, primarily due to economic reasons and funding related issues. Whether fundamental research should keep evolving independently from applied science, or whether it should regress to be nothing more than an auxiliary means of deciphering the mechanistic details of practical questions, remains to be established in the long run.

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